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13. ABSTRACT (Maximum 200 Words)

Homeobox genes represent a class of transcription factors important in embryogenesis, organogenesis, cell growth and differentiation, and cell migration. However, there is little known about their role in regulating endothelial cell (EC) phenotype in response to proangiogenic factors secreted by breast cancer, although at least two homeobox genes (*HOXD3* and *HOXD10*) have been implicated in inducing the angiogenic phenotype in ECs. We are therefore testing the hypothesis that the homeobox gene *Gax* regulates breast cancer-induced angiogenesis through its ability to regulate the expression of downstream target genes in ECs. Using in vitro tube formation assays, we have found that *Gax* expression inhibits *in vitro* angiogenesis. Moreover, by quantitative real time reverse transcriptase real time PCR, we have found that *Gax* expression is downregulated by proangiogenic factors, while cDNA micorarray analysis demonstrates that *Gax* downregulates pro-angiogenic adhesion molecules in ECs and upregulates the cyclin-dependent kinase inhibitor $p19^{INK4D}$. More importantly, *Gax* expression downregulates NF-κB activity in ECs. These observations will allow us to study the mechanism of *Gax*-mediated activation or repression of their expression to be studied and will form the basis for future studies that will examine in more detail the mechanism by which *Gax* activates downstream target genes and the detailed signaling pathways involved in this activation. Given the profound effect *Gax* has on endothelial cell activation, it is likely that these studies will identify new molecular targets for the antiangiogenic therapy of breast cancer. Ultimately, these same techniques will be applied to other homeobox genes implicated in regulating EC phenotype during breast cancer-induced angiogenesis.

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INTRODUCTION

Homeobox genes represent a class of transcription factors important in embryogenesis, organogenesis, cell growth and differentiation, and cell migration (1-6). However, there is little known about their role in regulating endothelial cell (EC) phenotype in response to pro- and antiangiogenic factors secreted by breast cancer cells. When we originally submitted our proposal, only two homeobox genes, *HOXD3* and *HOXB3*, had been implicated in regulating tumor-induced angiogenesis (2, 7, 8). Since then, three more (*HOXD10*, *HOXB5*, and *Hex*) have been added to the list of homeobox genes that influence the angiogenic phenotype in ECs (8-11). Of these three, two (*HOXD3* and *HOXD10*) have been directly implicated in regulating breast cancer-induced angiogenesis (12, 13). Because, of the handful of homeobox genes implicated in regulating angiogenesis, only *Gax* shows a strong restriction in its expression to cardiovascular tissues in the adult (14, 15), we originally proposed to test the hypothesis that *Gax* (14-30) regulates breast cancer-induced angiogenesis through its ability to regulate the expression of specific downstream target genes in vascular endothelial cells (ECs) We based this hypothesis on our preliminary data showing that *Gax* is expressed in vascular ECs and inhibits EC proliferation *in vitro*, later published as part of reference (19). Using a quantitative real-time PCR assay (31) and *in situ* hybridization (12, 13), we proposed to identify proangiogenic and antiangiogenic stimuli that determine *Gax* modulation and to examine the effect of breast cancer-secreted proangiogenic peptides and antiangiogenic therapies on *Gax* expression *in vitro* and in *in vivo* models of breast cancer angiogenesis. Next, using an adenovirus expressing *Gax* (26), we proposed to drive *Gax* expression in ECs in order to determine the effect of *Gax* expression on breast cancer angiogenesis, both *in vitro* and in *in vivo* models. Finally, because few downstream targets of *Gax* have been identified (25, 26, 29), we proposed to evaluate changes in global gene expression in ECs that result from *Gax* expression in order to identify and evaluate likely downstream targets. Our results were to form the basis for future studies that will examine in more detail the mechanism by which *Gax* activates downstream target genes and the detailed signaling pathways involved in this activation. Given the profound effect *Gax* has on EC activation, it is likely that these studies will identify new molecular targets for the antiangiogenic therapy of breast cancer.

BODY

Background

Like most cancers, breast malignancies are critically dependent upon inducing their ability to induce the ingrowth of blood vessels from the host in order to grow and metastasize (32, 33). Numerous studies have suggested a correlation between secretion of proangiogenic molecules and increased angiogenesis and increased likelihood of lymph node metastases with poorer prognosis in breast cancer (34, 35). Inhibition of tumor-induced angiogenesis has thus emerged over the last decade as a promising new strategy for breast cancer therapy, either alone or in combination with conventional therapies (36-39). Indeed, a recent ECOG study (E2100) it has been shown that the addition of the anti-vascular endothelial growth factor (VEGF) monoclonal antibody bevacizumab to paclitaxel improved disease free survival in patients with recurrent and metastatic breast cancer, so much so that the study was stopped and a press release issued (http://www.nci.nih.gov/newscenter/pressreleases/AvastinBreast). During angiogenesis, whether physiologic or tumor-induced, vascular ECs undergo distinct changes in phenotype and gene expression, including activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix (40-42). Although the EC receptors and signaling pathways activated by proangiogenic factors secreted by breast cancer cells, such as vascular endothelial growth factor (VEGF) (43, 44) and basic fibroblast growth factor (bFGF) (43), have been extensively studied (45-47), much less is known about the molecular biology of the downstream transcription factors activated by these signaling

pathways, which then activate the genes necessary for EC phenotypic changes during breast cancer-induced angiogenesis.

Homeobox genes encode transcription factors containing a common DNA-binding motif (1, 4-6, 48). Important regulators of body plan and cell fate during embryogenesis, homeobox genes also have pleiotropic roles in many cell types in the adult and can modulate cell cycle progression and arrest, cell differentiation, migration, and apoptosis (1, 3-5, 7, 19, 49, 50). As a gene family, they are thus excellent candidates to be involved in the final transcriptional control of genes responsible for the changes in EC phenotype induced by breast cancer-secreted proangiogenic factors. Until recently, little was known about how homeobox genes might influence angiogenesis. There is now evidence for their involvement in phenotypic changes ECs undergo during angiogenesis, and, in particular, during breast cancer angiogenesis (7, 8, 10, 12, 19). For instance, one homeobox gene, *HOXD3*, induces the expression of $\alpha_V\beta_3$, an integrin important in angiogenesis (51), resulting in the conversion of ECs to an angiogenic phenotype both *in vitro* and *in vivo* (7); impaired *HOXD3* expression is associated with impaired angiogenesis in a mouse model (50) and increased *HOXD3* expression is observed in the vasculature of breast cancer and DCIS compared to the vasculature of the surrounding normal breast (13). Similarly, overexpression of the homeobox gene *HOXB3* results in an increase in capillary vascular density and angiogenesis (8). Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating downstream genes to induce phenotypic changes associated with breast cancer-induced angiogenesis. More recently, three additional homeobox genes have been implicated in the regulation of EC phenotype during angiogenesis. First, in contrast to *HOXB3* and *HOXD3*, another HOX cluster gene (*HOXD10*) inhibits EC conversion to the angiogenic phenotype (12). *HOXD10* expression is elevated in quiescent vascular endothelium in the stroma compared to breast cancer-associated vascular endothelium (12). Consistent with these observations, *in vivo* human ECs overexpressing *HOXD10* fail to form new blood vessels when embedded in sponges containing Matrigel and proangiogenic factors (12) in nude mice. Another homeobox gene, *HOXB5*, transactivates the *flk-1* promoter and leads to the expansion of flk-1-positive angioblasts in embryonic development (11). Finally, *Hex* expression in human umbilical vein endothelial cells (HUVECs) inhibits angiogenesis and blocks VEGF receptor signaling (9, 10). We anticipate that more homeobox genes that regulate the angiogenic phenotype will be described in the future.

The cardiovascular-specific homeobox gene *Gax* appears more likely to function as a negative regulator of breast cancer-induced angiogenesis in ECs, like *HOXD10* or *Hex*. After isolating it from a rat aorta cDNA library (14, 52), we and others have shown that *Gax* has profound effects on cardiovascular tissues (18, 19, 21, 22, 24-26, 29). In vascular smooth muscle cells (VSMCs) *Gax* expression is downregulated by mitogenic signals and upregulated by growth arrest signals (14, 30). Consistent with this observation, *Gax* induces G_1 cell cycle arrest (26) and can induce apoptosis in VSMCs under stress (24). Also, *Gax* overexpression inhibits VSMC migration, downregulating the expression of integrins, $\alpha_V\beta_3$ and $\alpha_V\beta_5$, both of which are associated with the activated ("synthetic") state in VSMCs and the angiogenic phenotype in ECs (29, 51). *In vivo*, *Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury (21, 22, 25, 26). Based on these observations in VSMCs, we looked for and found evidence that *Gax* mRNA is also expressed in ECs (19). Understanding the actions of *Gax* on downstream target genes, as well as signals that activate or repress *Gax* expression, could thus lead to a better understanding of the mechanisms of breast cancer-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of breast cancer. Thus, the studies we have proposed and undertaken with support from the Department of Defense have attempted to use *Gax* as a molecular tool to: (1) enhance our understanding of the mechanisms by breast cancer stimulates endothelial cells to become angiogenic; and (2) provide the basis for the design of antiangiogenic therapies of breast cancer targeting *Gax* or its downstream targets.

Progress overview

Since this project began, we have made considerable progress in meeting the milestones originally proposed in our original Statement of Work. Of particular interest, in analyzing our early cDNA microarray experiments, we made the unexpected observation that *Gax* expression downregulates NF-κB-dependent gene expression in ECs (see Task #6). This observation has suggested an entirely new area of research into the mechanism by which *Gax* expression inhibits angiogenesis, as there is now considerable evidence that NF-κB activity is proangiogenic in ECs. Consequently, during Year Two, we formally requested a change in our Statement of Work, which was granted. We are therefore presenting our final report in relation to the modified Statement of Work.

List of personnel:

	Role	%Effort
David H. Gorski, MD, PhD	Principle investigator	50%
Sejal Patel, PhD	Investigator	60% (no salary support)
Alejandro Leal	Technician	100% (no salary support)

Detailed progress report by tasks in the modified Statement of Work

Task 1: ***Characterize the regulation of Gax expression in three different endothelial cell types in vitro, months 1 to 24:***

a. **Develop and verify real time quantitative reverse transcriptase polymerase chain reaction assay to measure *Gax* transcript levels. (Months 1-6.)**

Status: Completed.

Results and Discussion: This was initially discussed in detail in our Annual Report for Year One (2003). We have successfully developed a quantitative real time PCR assay to measure *Gax* mRNA levels on schedule. Initially, we used SYBR Green as our detection method in preliminary experiments. However, melting curves performed using a variety of *Gax*-specific primer sets demonstrated that primer-dimer is frequently present at levels that severely interfere with interpretation of data, especially given the limitations of the software suite that came with our equipment (data not shown). Because *Gax* message is of low abundance, the presence of primer-dimer can potentially compromise our accuracy. Therefore, we began to utilize TaqMan probes. We generally used carboxyfluorescein fluorescent dye 6-FAM as the 5'-fluorophore and Black Hole Quencher-1 (BHQ-1, Biosearch Technologies, Inc.) as the 3'-quencher.

Primer and probe design. For our proposed experiments involving real time quantitative PCR, we used the MacVector v.7.1 DNA analysis software package to design specific primers and TaqMan probes. The primer/probe set that we are currently using to measure *Gax* transcript amplifies a 238 bp sequence of the human *Gax* coding sequence (14, 20) between bases 803 and 1040. The probe binds to bases 962 to 982 and has a calculated melting point of 69.5° under the reaction conditions used. We normalize to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using primers that amplify 138 bp fragment from 572 to 709 of the human gene and a probe that binds from 625 to 644 (53). Before the primers and probes were synthesized, their sequences were subjected to a BLAST (54, 55) search against the Genbank database, in order to detect any possibility that they might bind to or amplify genes other than the ones for which they were designed. Further, all reactions were subjected to agarose gel electrophoresis, to verify that the PCR reaction products were of the correct size.

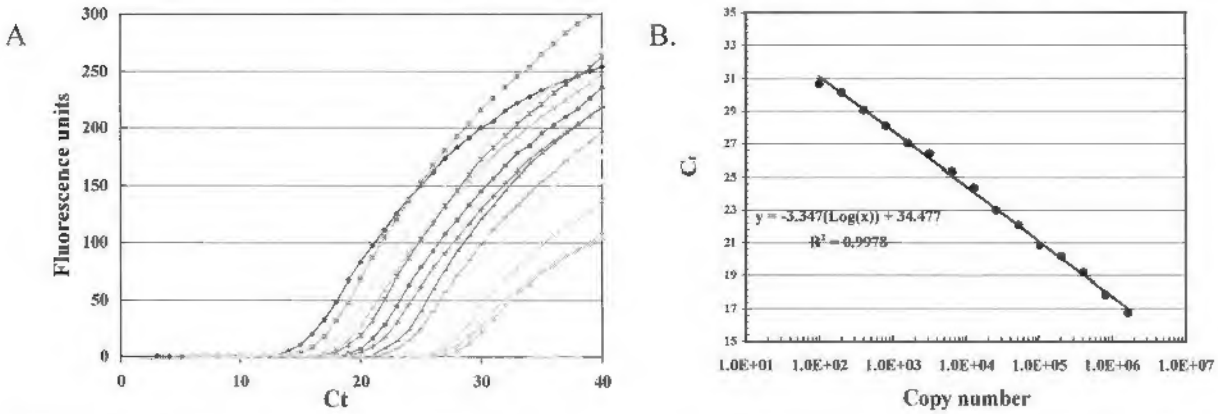


Figure 1. Representative real time PCR standard curve for *Gax*. Using primers and a TaqMan probe (5' end=FAM, 3' end=BHQ1) specific for *Gax*, serial dilutions of the Gax cDNA from 1.64 million to 100 copies were subjected to real time quantitative PCR and a standard curve produced. There was an excellent linear fit to the semilog curve (r^2=0.998). The calculated amplification efficiency from the standard curve slope was 98.9%.

RNA isolation and reverse transcriptase polymerase chain reaction (RT-PCR). Before running assays on experimental samples, each primer/probe set, annealing conditions, Mg^{2+} concentration, and primer and probe concentration were optimized using plasmids containing the *Gax* cDNA (14). We were able to detect as few as 10-100 copies of the *Gax* cDNA in our assays. Because the *Gax* gene has a single exon (20), all RNA samples were treated with RNA-free DNAse prior to reverse transcription, and random RNA samples are subjected to sham reverse transcription (no reverse transcriptase) and real time PCR with the GAPDH primer/probe set, to verify that there was no genomic DNA contamination. The PCR cycle consisted of an initial 2.5 minute denaturation step at 95° C, followed by 40 cycles of denaturation at 95° C for 15 seconds, annealing at the appropriate annealing temperature for each primer for 15 to 30 seconds, and extension at 72° C for 15 to30 seconds, with exact conditions depending upon the specific probe/primer set.

Normalization and quantification. In our preliminary experiments, we estimated relative *Gax* levels by calculating the differences in threshold cycle (C_t) between *Gax* and our control gene ($\Delta Ct = C_t^{GAPDH} - C_t^{Gax}$) and used the formula: *Gax* level $\approx 2^{-\Delta Ct}$. (For results of these experiments, see Task 1b, below.) While this method is useful for estimating relative levels of a gene and changes in expression, it depends upon the assumption that the PCR efficiency is identical for the *Gax* and GAPDH primer sets (31, 56). While this is approximately true for *Gax* and GAPDH (data not shown), it may not be true for primer sets designed for other genes we proposed to examine. For future experiments we have developed a more rigorous method. Using the primers from the TaqMan probe/primer sets, we amplify specific PCR products for RNA samples known to be positive for the gene of interest by conventional PCR. These are then subjected to electrophoresis on agarose gels and the specific PCR product bands cut from the gel and extracted using Qiaex II (Qiagen, Inc.). The fragments were serially diluted in log steps from 10^8 copies to 10 copies in a 1 μl volume and amplified in real time PCR reactions. Calibration curves were then constructed by making a semilog plot of C_t versus the known copy number for each plasmid. In Figure 1, we present a representative real time PCR experiment, in which a standard curve for *Gax* has been constructed by two-fold serial dilutions of the full length *Gax* cDNA.

b. Measure changes in *Gax* mRNA levels in three different endothelial cell types in response to growth factors, pro-angiogenic, and antiangiogenic factors. (Months 6-24)

Status: Completed.

Results and Discussion: Using our quantitative real time reverse transcriptase PCR assay, we completed the *in vitro* experiments and found that, for nearly every breast cancer cell line we have studied,

serum-free media conditioned for 24 hours by breast cancer cells strongly downregulated *Gax* expression in ECs within four hours. Two cell lines, MCF7 and MDA-MB231, were as potent as fetal bovine serum in downregulating *Gax* (Figure 2).

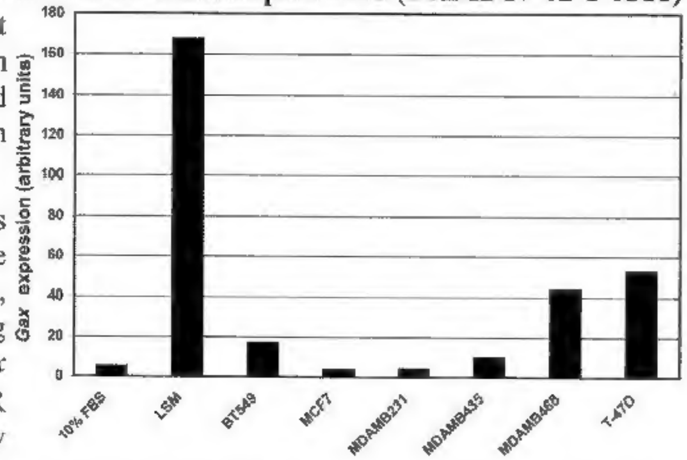


Figure 2. Downregulation of *Gax* expression in endothelial cells by conditioned medium from tumor cell lines. Quiescent HUVECs were treated with either low serum medium (LSM), 10% FBS, or 10% conditioned medium from the indicated breast cancer cell lines. Cells were harvested 4 hours after stimulation, total RNA harvested and real time quantitative RT-PCR performed. *Gax* message level was normalized to GAPDH. Units are arbitrary.

To begin to identify the specific factors secreted by breast cancer cells that are likely to be the ones that result in downregulation of *Gax* expression, we have followed up these observations by examining the effect of VEGF, bFGF, and TNF-α on *Gax* message levels using quantitative real time PCR (Figure 2). In all cases, *Gax* was rapidly downregulated and then more slowly returned to baseline after stimulation with proangiogenic factors. First, we studied the time course of *Gax* downregulation. HUVECs made quiescent by incubation for 24 hrs in 0.1% FBS were stimulated with 10% FBS. *Gax* was rapidly downregulated by more than 5-fold within four hours and slowly returned to basal over 24 hours (Figure 3A). Conversely, when sparsely plated randomly cycling HUVECs were placed in medium containing 0.1% serum, *Gax* was upregulated nearly 10-fold within 24 hours. We then stimulated quiescent HUVECs with proangiogenic or proinflammatory factors, including bFGF, VEGF, and TNF-α. *Gax* was rapidly downregulated with a similar time course (Figure 3, B through D). Similar results were observed in HMEC-1 cells, an immortalized human microvascular endothelial cell line (57) and HPMEC1.6R (58). So far, the results are similar to those shown in Figure 3.

Finally, we examined whether antiangiogenic peptides that might be used either alone or in combination (59, 60) to treat breast cancer affected *Gax* expression. Randomly cycling HUVECs were incubated for varying times with 1 μg/ml angiostatin (59) or endostatin (60). Cells were harvested for total RNA isolation and the RNA then subjected to quantitative real time PCR to measure *Gax* expression. We found that both angiostatin and endostatin upregulated *Gax* expression by two-fold over 48 hours, a time course that was slower and an upregulation that was less dramatic than that caused by serum deprivation (Figure 4). Thus far, we have not been able to find a growth stimulus that does not downregulate *Gax* or a growth arrest stimulus that does not upregulate it. Thus, the promoter mapping experiments originally proposed will now be given a higher priority in future experiments.

c. **Measure changes in *Gax* mRNA levels *in vitro* using three different endothelial cell types in response to common cytotoxic therapies used in breast cancer, including chemotherapy and radiation. (Months 12-36.)**

Status: Incomplete.

Results and Discussion: These experiments were deferred in order to study more closely the interaction between *Gax* and NF-κB (see Task #6). We began these experiments recently, starting with the antiangiogenic factors angiostatin and endostatin, with the plan being to proceed to cytotoxic therapies this summer. We do not have reportable data from these experiments.

d. **Mechanistic studies to determine if regulation of *Gax* expression occurs at the level of transcription, translation, or mRNA stability and mapping of the *Gax* promoter, if necessary. (Months 12-36.)**

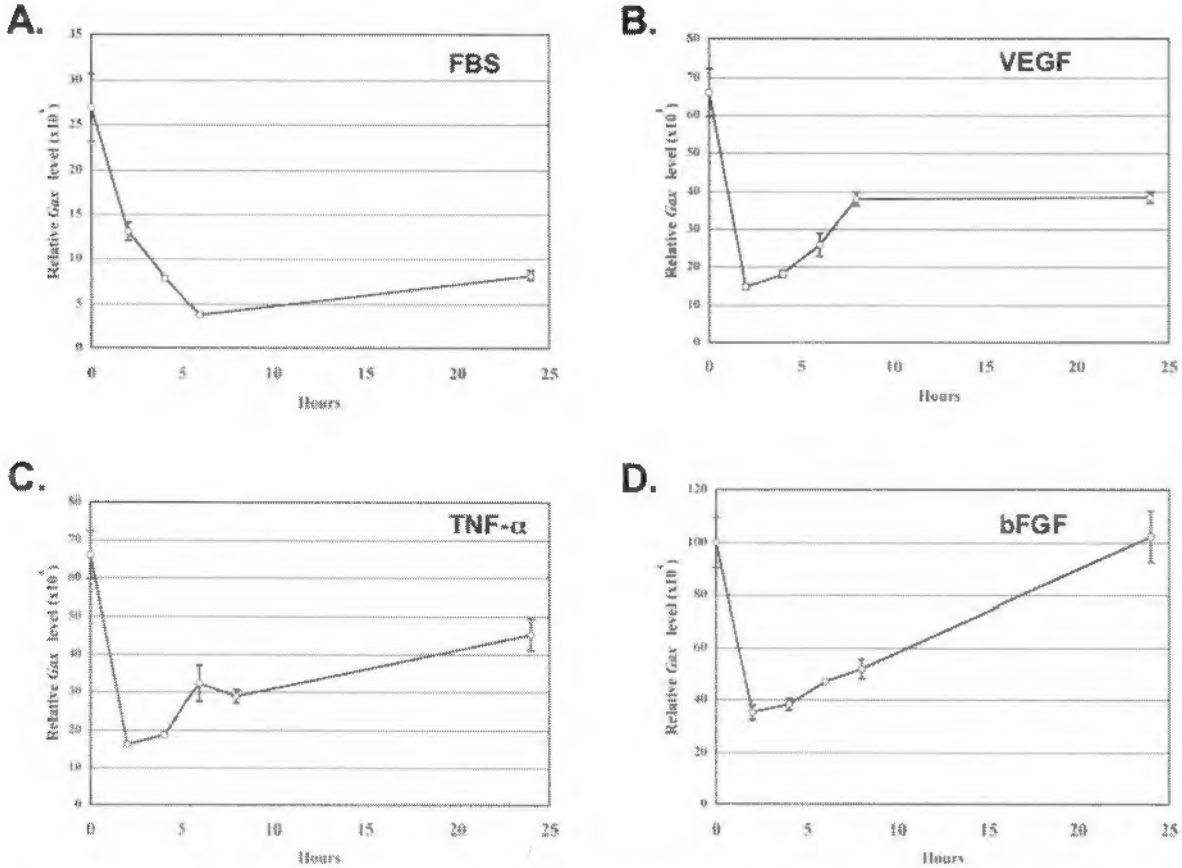


Figure 3. Time course of *Gax* downregulation by mitogens and proinflammatory factors. Quiescent HUVECs were treated with either **(A)** 10% fetal bovine serum (FBS); or 10 ng/ml of either **(B)** $VEGF_{165}$; **(C)** TNF-α; or **(D)** bFGF. At various time points, cells were harvested for extraction of total RNA, which was then subjected to quantitative real time TaqMan RT-PCR with *Gax*- and GAPDH-specific primer/probe sets. *Gax* mRNA levels were normalized to GAPDH. Units are arbitrary. E. Cells were treated identically to **(C)**, except that after six hours cells were harvested for protein extraction and then subjected to Western blot with Gax-specific polyclonal antibody.

Status: Incomplete.

Results and Discussion: As of the conclusion of this grant, these experiments are incomplete. These experiments were also deferred in order to study more closely the interaction between *Gax* and NF-κB (see Task #6) and are therefore behind schedule. We are presently in the early stages of doing these experiments, having recently obtained a series of *Gax* promoter deletion constructs (16) using Luciferase as the reporter gene to use to identify important elements for regulating *Gax* expression. We are also constructing additional *Gax* promoter constructs with additional deletions.

<u>Task 2</u>:***Measure differences in Gax expression between angiogenic blood vessels and normal blood vessels in vivo. (Months 13 to 36.)***

a. **Measure breast cancer cell line-induced angiogenesis *in vivo* using the Matrigel plug assay and breast cancer cell line-conditioned media, and measure *Gax* expression in endothelial cells *in vivo*. (Months 13-36.)**

Status: In progress.

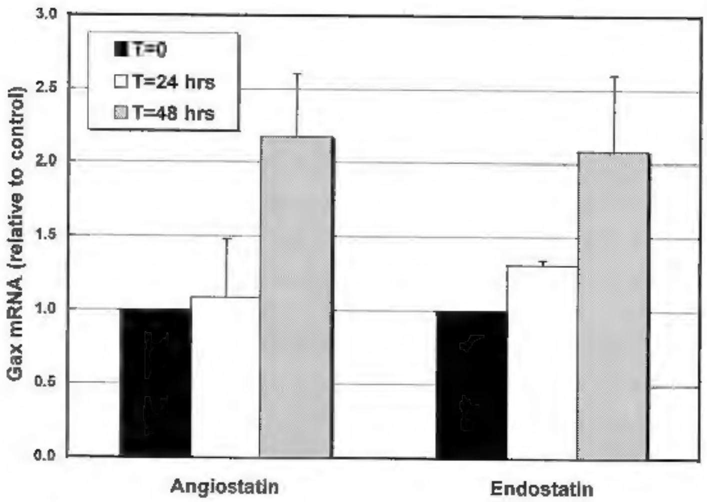


Figure 4. Upregulation of Gax by antiangiogenic peptides. Randomly cycling HUVECs were treated with either angiostatin or endostatin at 1 μg/ml. At varying time points, cells were harvested for RNA isolation, which was then subjected to reverse transcriptase quantitative real time PCR. Gax mRNA levels were normalized to GAPDH and expressed as ratios to Gax levels in control HUVECs allowed to incubate in parallel in normal medium. $p<0.01$ at 48 hrs for angiostatin and endostatin.

Results and Discussion: These results will be discussed together with the results of Task #2b. The experiments described are preliminary experiments in which we have been working out the conditions for our in situ hybridization and immunohistochemistry of frozen tissue sections. See below for a combined discussion.

b. **Compare immunohistochemical staining for *Gax* expression in breast tumor blood vessels with that of blood vessels found in normal breast for 50 invasive human breast cancer specimens. (Months 13-36.)**

Status: In progress.

Results and Discussion: In order to determine if *Gax* expression *in vivo* varies according to the angiogenic state of the EC, we measured *Gax* expression *in vivo* in frozen sections of normal human breast and in human breast cancer by *in situ* hybridization. We also measured Gax protein expression in the mouse tissues from Matrigel plug experiments. In initial preliminary experiments, we observed *Gax* message expression in the capillaries and blood vessels from normal human colon (Figure 5) using *in situ* hybridization and succeeded in detecting Gax protein in normal human arteries (19). We then studied normal breast tissue (Figure 6, A and B). More interestingly, in a human breast cancer specimen (Figure 6C) we could also detect *Gax* expression in capillaries in the surrounding normal stroma. However, we found very few capillaries or blood vessels in the tumor itself expressing *Gax*. Consistent with this, by immunohistochemistry in frozen sections we were able to detect Gax expression in blood vessels in the skeletal muscle (Figure 6D) and stroma surrounding the Matrigel plugs (Figure 6, E and F). In contrast, the neovessels we found in the Matrigel plugs either stained weakly for Gax or not at all. We caution that these results are preliminary and considerable work remains to determine whether *Gax* message and protein expression is indeed lower in angiogenic vasculature or in breast cancer vasculature than in resting vasculature. In particular, we need to define more carefully what represents positive staining for *Gax* and then quantify the number of vessels staining positive for *Gax*. This may require double-staining with antibodies to vascular-specific markers, such as CD31. Also, the frozen sections we obtained from our Tissue Retrieval Service were too thick, hence the poor tissue and cellular definition in Figure 6, A through C. These caveats aside, however, these data do at least suggest that *Gax* is regulated *in vivo* in a manner similar to how it is regulated *in vitro*, further implying a role for *Gax* in regulating *in vivo* angiogenesis. Although this study is now finished, we will still use these preliminary results to determine whether *Gax* expression is downregulated *in vivo* by breast cancer-secreted angiogenic factors and whether its expression is truly downregulated *in vivo* in breast cancer- and DCIS-associated vasculature, as originally proposed, using R01 funding from the NCI obtained based largely on the strength of preliminary data from this project.

<u>Task 3</u>: ***Determine the effects of Gax overexpression in ECs in vitro. (Months 1-24.)***

a. **Determine effect of *Gax* overexpression on endothelial cell proliferation and expression of cell cycle regulatory genes. (Months 1-12.)**

Status: In progress.

Results and Discussion: Using cDNA microarray experiments, we have identified several cyclin dependent kinase inhibitors that are upregulated by *Gax* expression, including $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF1/CIP1}$ (26, 61, 62). These experiments will be described in more detail in Task #4. The upregulation of these CDK inhibitors suggests redundant mechanisms by which *Gax* can induce G_1 cell cycle arrest. In Year One, we had also shown that the upregulation of p21 in ECs is due to a p53-independent activity of *Gax* on the $p21^{WAF1/CIP1}$ promoter (19). We are presently verifying the upregulation of $p57^{Kip2}$ and $p19^{INK4D}$ and looking at additional cell cycle regulatory proteins.

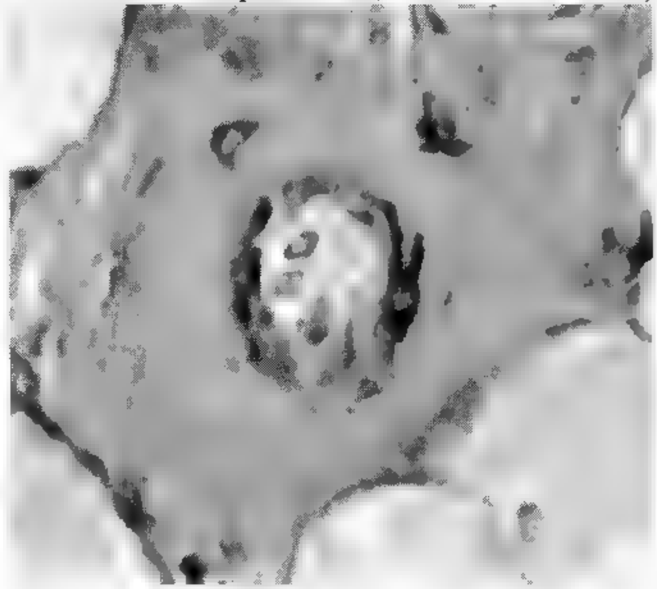


Figure 5. *Gax* expression in tumor blood vessels. PCR was used to generate a 329 bp fragment of the *Gax* cDNA (nucleotides 549 to 877), which was then used to generate a riboprobe for *in situ* hybridization. This probe was used to label sections of normal human colon. This tumor blood vessel stained positive for *Gax* expression. Sense probe did not demonstrate any staining (not shown)

b. **Determine effect of *Gax* overexpression on expression of integrins, specifically if the expression of integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ are regulated by *Gax* expression (Months 18-36.)**

Status: In progress.

Results and Discussion: Migration of ECs through the basement membrane and into the surrounding stroma in response to proangiogenic stimuli is a critical step in tumor-induced angiogenesis, and integrins, particularly integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$, play a critical role in this process (51), as might integrin $\alpha_5\beta_1$, which has been implicated in both *HOXD3*-induced (13) and NF-κB-mediated angiogenesis (63). We therefore tested the ability of *Gax* to inhibit EC migration towards proangiogenic factors. HUVECs were transduced with Ad.r*Gax* or Ad.h*Gax* at varying MOI and incubated overnight. 10^5 viable cells per well were plated in 6-well plates with inserts containing 8 μm polycarbonate filters, and we measured their migration towards serum-containing media in the lower chamber. Ad.r*Gax* strongly inhibited the migration of HUVECs towards serum, VEGF, bFGF, and TNF-α (Figure 7), as did Ad.h*Gax* (data not shown). Both homologs also inhibited migration of HMEC-1 cells towards bFGF and VEGF (data not shown).

We have recently begun to examine the levels of integrin subunits in ECs and how they change in response to *Gax* expression. Our strategy will use real time quantitative reverse transcriptase PCR, Western blotting, and flow cytometry to measure changes in integrin expression induced by *Gax* expression. An initial flow cytometry experiment showed no change in the cell surface level of integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ in response to *Gax* expression using our adenoviral vectors (data not shown). Given that this result conflicts with other data in vascular smooth muscle cells (29), we are presently attempting to repeat these experiments and determine if this result is correct. If it is correct, it would imply a cell type-specific difference in how *Gax* regulates integrin expression and suggest that the mechanism through which *Gax* inhibits angiogenesis does not involve integrin $\alpha_V\beta_3$ or $\alpha_V\beta_5$.

c. **Characterize *Gax*-induced endothelial cell apoptosis and the effect of *Gax* on the expression of genes regulating apoptosis. (Months 24-36.)**

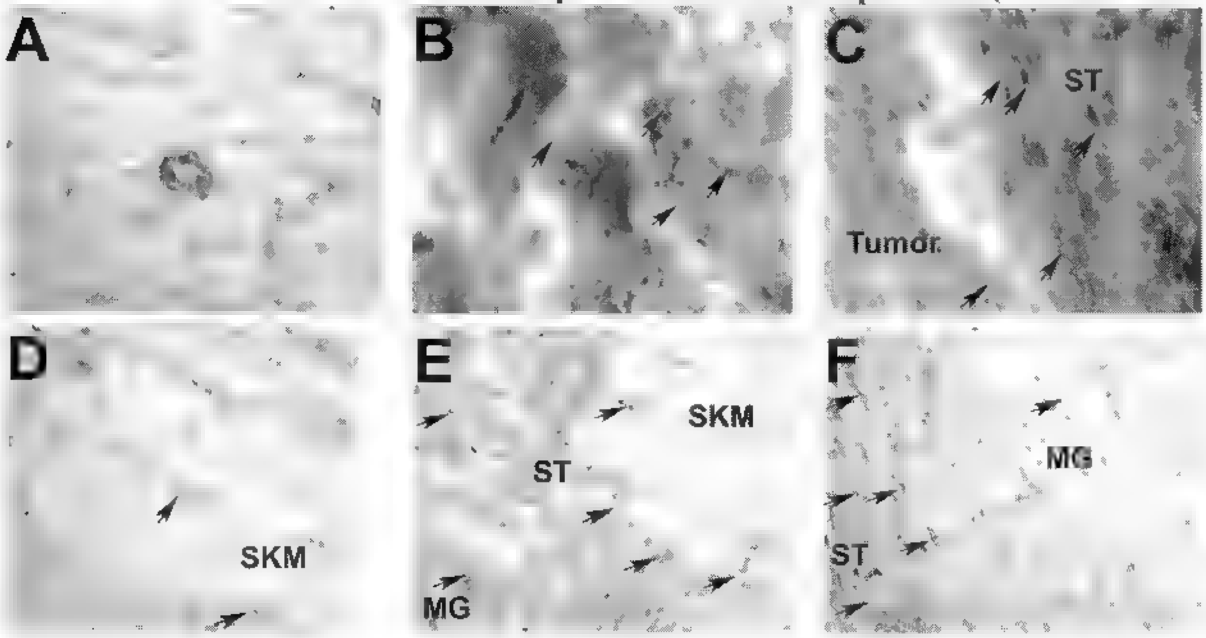


Figure 6. Determination of *Gax* expression *in vivo*. *Gax* expression was measured in human breast and breast cancer specimens by *in situ* hybridization with a riboprobe for *Gax* as described in the original grant in Specific Aim #3, p 45 (A through C) and in Matrigel plugs harvested from mice by immunohistochemistry on frozen sections with previously described anti-Gax antibody (D through F). All photographs were taken at 400x magnification. Arrows indicate blood vessels or capillaries staining positive for *Gax* expression (Legend: ST=stroma; SKM=skeletal muscle, MG=Matrigel plug.) **A. Normal breast (*in situ* hybridization).** In the fatty tissue of a normal human breast, a blood vessel is observed to stain positive for Gax expression. **B. Normal breast (*in situ* hybridization).** Several capillaries stain positive for *Gax* expression. **C. Breast cancer (*in situ* hybridization).** Multiple capillaries in the stroma stain positive for *Gax* expression. However, capillaries in the tumor either stain much more weakly or do not stain positive for *Gax* at all. **D. Mouse skeletal muscle (immunohistochemistry).** Blood vessels in the skeletal muscle near a Matrigel plug stain positive for Gax expression. **E and F. Immunohistochemistry of control Matrigel plugs (bFGF only, no virus).** Blood vessels in the surrounding skeletal muscle or connective tissue stroma stain strongly for Gax expression, but vessels noted within the Matrigel plugs, where angiogenesis is occurring, stain either weakly or not at all.

Status: Not completed.

Results and Discussion: This task was not begun prior to the end of the grant period.

<u>Task 4</u>: ***Determine the effects of Gax overexpression on angiogenesis in vivo. (Months 13-36.)***

a. **Matrigel plug assays in C57BL/6 mice to determine if Ad.*Gax* inhibits *in vivo* angiogenesis and to quantify how strong the effect is. 100 mice will be required. (Months 13-36)**

Status: In progress

Results and Discussion: Matrigel containing proangiogenic factors, when implanted subcutaneously in mice, can stimulate the ingrowth of blood vessels into the Matrigel plug from the surrounding tissue, and this neovascularization can be estimated by counting CD31-positive cells and/or by measuring hemoglobin concentrations in the plug (64). Moreover, adenoviral vectors diluted in Matrigel implanted as subcutaneous plugs can serve as reservoirs to transduce ECs invading the plug and drive expression of exogenous genes (65, 66), producing effects on *in vivo* angiogenesis even when the gene transduced is a transcription factor (67). As originally proposed, we have taken advantage of this observation to test whether exogenously driven *Gax* expression can inhibit angiogenesis *in vivo*, using methodology previously described (65, 66). Matrigel plugs containing bFGF and either Ad.GFP, Ad.*hGax*, or Ad.*rGax* were injected subcutaneously in C57BL/6

mice (N=8 per experimental group). As a positive control for angiogenesis inhibition by a viral vector, we utilized an adenoviral construct expressing a dominant negative form of Akt (Ad.DN-Akt) (65, 66). We observed that the adenoviral vectors expressing *Gax* expression inhibit the neovascularization of the plugs with a potency slightly less than that observed for the Ad.DN-Akt construct (Figure 8), and that the Ad.DN.Akt construct inhibited neovascularization with a potency similar to what has previously been reported (65, 66). The results of these experiments indicate that *Gax* is capable of inhibiting angiogenesis in *in vivo* models.

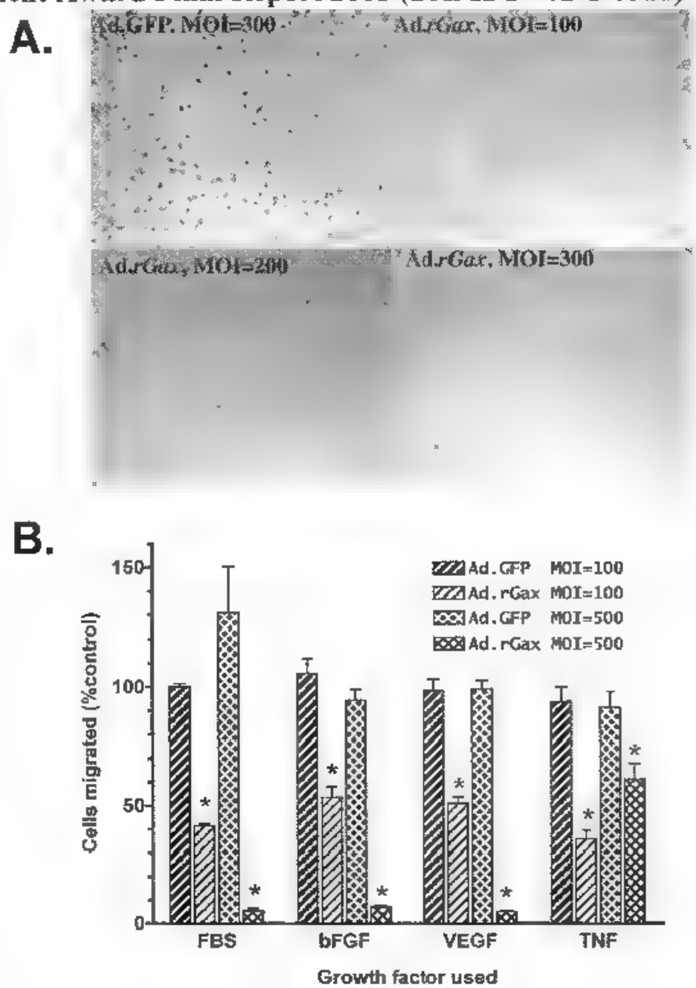


Figure 7. ***Gax*** **inhibits HUVEC migration towards serum.** HUVECs were transduced with varying MOI of either Ad.*GFP* or Ad.*rGax* and their migration towards various growth factors and proangiogenic factors determined. *Gax* inhibits HUVECs migrating towards **(A)** FBS; and **(B)** FBS, bFGF, $VEGF_{165}$, and TNF-α. Results are expressed relative to control HUVECs not transduced with any virus. Results were analyzed by one-way ANOVA (* indicates $p<0.01$). Similar results were obtained with Ad.*hGax* (data not shown).

Task 5: ***Identify potential downstream targets of Gax. (Months 1 through 24.)***

a. Compare global gene expression between *Gax*-expressing endothelial cells and non-*Gax*-expressing endothelial cells using cDNA microarrays. (Months 10 to 18.)

Status: In progress.

Results and Discussion: We reported preliminary results of our cDNA microarray experiments, in which we compared the global gene expression of HUVECs transduced with Ad.r*Gax* and Ad.h*Gax* with that of HUVECs transduced at an equal MOI with an adenoviral vector expressing GFP (Ad.GFP) in last year's Annual Report (2004). Since then, we have concentrated primarily on two tasks: (1) analyzing the data and verifying the regulation of downstream targets by real time PCR and/or Western blot; and (2) continuing to try to develop stable transfectants with tetracycline-inducible *Gax* expression as a strategy by which we will be able to manipulate *Gax* at more physiologically relevant expression levels that what is driven by adenoviral constructs and to allow time courses of changes in EC phenotype and gene expression secondary to *Gax* expression.

We have now successfully generated several clones based on HMEC-1 cells with the Tet-On system (Clontech). These cells have varying levels of doxycycline-inducible gene activity when plasmids containing the Luciferase gene under control of the Tet response element (TRE). Using the Tet-On system, we have generated HMEC-1 clones with constitutive expression of rTA. When these cells are transduced with a reporter construct in which Luciferase is driven by the Tet response element (TRE), expression of reporter gene is induced by exposure to doxycycline (Figure 9A). There are several candidate clones with tetracycline-inducible expression, the most promising of which is clone #26. This clone will be transfected with pTRE-*Gax*, a construct in which expression of the *Gax* cDNA is controlled by TRE to produce tetracycline-inducible *Gax* expression. However, efforts to complete the second step and stably transfecting

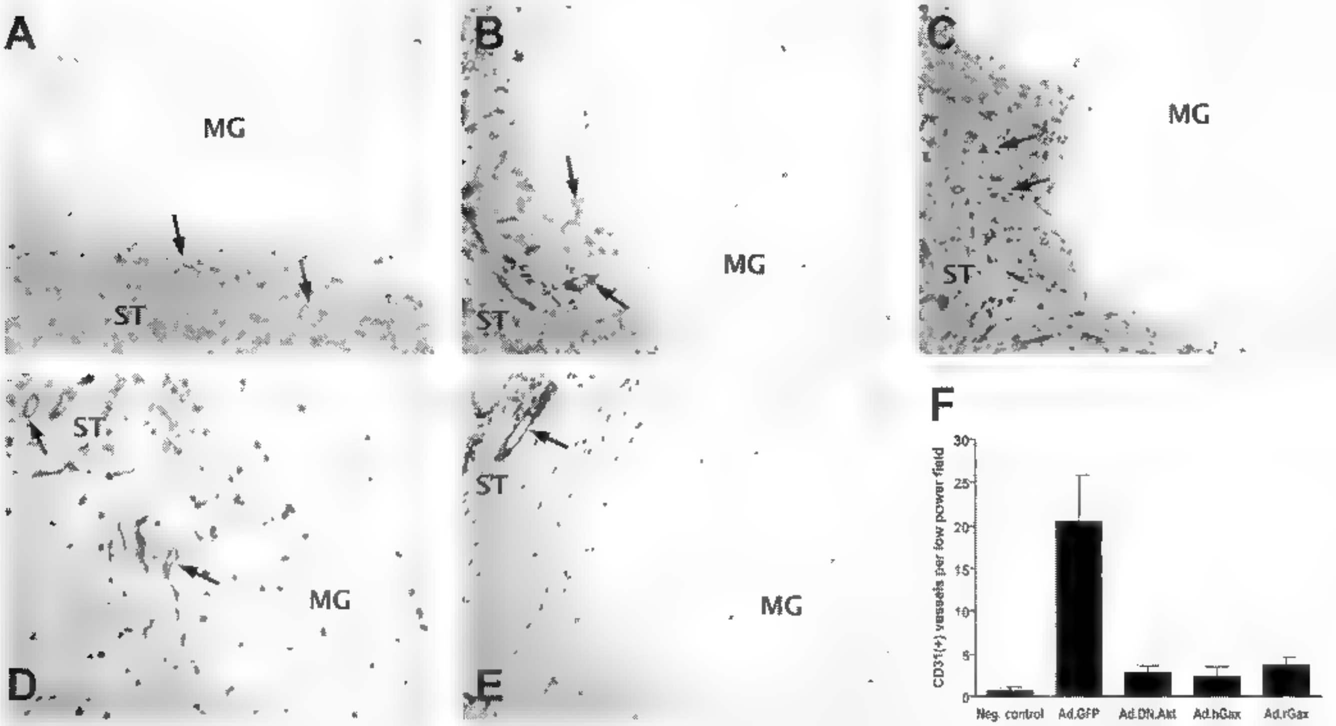


Figure 8. Effect of *Gax* expression on angiogenesis in Matrigel plugs. Matrigel plugs (500 ul each) containing 400 ng/ml bFGF and viral constructs at the pfu indicated were implanted subcutaneously in the flanks of C57BL6 mice (N=3 per experimental group). Differences in pfu/plug in different experimental groups are due to low titers of our Ad.r*Gax* and Ad.DN.Akt viral stocks. In future experiments, viral stocks will be prepared so that equal titers of at least 10^8 pfu/plug are achieved and dose-response experiments are possible. Plugs were harvested after 14 days incubation for immunohistochemistry using CD31 antibodies (see text for details) and counterstained with light blue. Slides were photographed at 200x magnification. (**Legend:** MG = Matrigel plug, ST = stroma surrounding the plug; arrows indicated examples of CD31-positive blood vessels.) **A.** Ad.DN.Akt, 5×10^7 pfu/plug. **B.** Ad.h*Gax* . x 10^8 pfu/plug. **C.** Ad.r*Gax*, 2.0×10^7 pfu/plug. **D.** Ad.GFP 1.0×10^8 pfu/plug (positive control), note the infiltration of the plug with CD31-positive vessels such that it is difficult to determine the exact edge of the plug. **E.** Negative control (no virus, no bFGF). **F.** Vessel counts. CD31-positive vessels were counted and the number of vessels per low-powered field determined for each plug. Vessels were counted only at low powered fields immediately adjacent to the edge of a Matrigel plug, and four low-powered fields per plug were counted. Results are plotted as vessel count ± S.E.M. Differences were calculated with one-way ANOVA $p=0.01$ for the overall, and the vessel counts were statistically significantly different for Ad.DN.Akt ($p=0.013$); Ad.h*Gax* ($p=0.033$), and Ad.r*Gax* ($p=0.028$).

HMEC-1/rTA line with the best tetracycline-inducible gene expression with TRE-*Gax* and producing a stably transfected HMEC-1 clone with tightly inducible *Gax* expression by tetracycline have thus far failed. Consequently, we tried a different method to generate HMEC-1 clones with inducible *Gax* expression using an ecdysone-inducible system (Invitrogen) (68). We have now produced several stable transfectants with Ponasterone A-inducible gene expression (Figure 9B), but have not yet produced a stable cell line with Ponasterone A-inducible *Gax* expression. Despite our initial success in this first step of stably expressing rTA, it is still possible that we may not be able to develop stable transfectant HMEC-1 cells with inducible *Gax* expression using this system or these cells. In this event, we will pursue two additional strategies. First, we have obtained another EC cell line, HPMEC-ST1.6R (69), which we are presently expanding for use in generating stable transfectants with inducible *Gax* expression. Second, we will consider using a retroviral system (such as the BD RevTet-On vector (BD Biosciences, Palo Alto, CA) to generate either stable transfectants with inducible *Gax* expression or to generate long term transient inducible *Gax* expression whose duration should be adequate to do the experiments originally proposed.

b. **Data analysis of cDNA microarray data to identify putative downstream targets of *Gax*. (Months 19-24.)**

   Status: Completed.

Results and Discussion: We examined genes that were downregulated 24 hours after transduction of HUVECs with Ad.r*Gax* and were immediately struck by the number of CXC chemokines strongly downregulated (Table 1 and Ref. (70)). These results were reported in last year's Annual Report, but, because the experiments had been done immediately before the report was due, we had had little time to analyze them. Most strongly downregulated of all was GRO-α (CXCL1), a CXC chemokine and a growth factor for melanoma that has also been implicated in promoting angiogenesis (71). Similarly, several other CXC chemokines were also strongly downregulated by *Gax* expression. Many of these peptides are clearly important in mediating EC activation during inflammation and in promoting angiogenesis (72). Consistent with the hypothesis that *Gax* inhibits EC activation, we also observed the downregulation of several cell adhesion molecules known to be upregulated in ECs during activation and angiogenesis, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and E-selectin (73, 74). These proteins have all been implicated in leukocyte-EC interactions and are upregulated by pro-inflammatory factors and by VEGF during angiogenesis (73). The pattern of downregulation of these adhesion molecules, coupled with the downregulation of CXC chemokines, suggested to us inhibition of genes normally induced by TNF-α, which in turn suggested the possibility that *Gax* may inhibit nuclear factor κB (NF-κB) activity. Indeed, when we examined our data using GeneMAPP to look for patterns of signal-dependent gene regulation (75), we found numerous NF-κB-dependent genes (76) downregulated 24 hrs after *Gax* expression (Table 1). These data strongly implied that *Gax* somehow interferes with NF-kB activity in ECs. Given that NF-κB signaling has been implicated in angiogenesis, particularly through paracrine stimulation (77) and maintenance of EC survival pathways (78), these data also suggest a potential mechanism by which *Gax* inhibits angiogenesis.

The genes upregulated by *Gax* did not fall into any signal-dependent patterns as striking as the pattern of genes downregulated by *Gax*. However, we did note results that might suggest specific pathways upregulated by *Gax*. First, there was a strong upregulation of ALK3 (bone morphogenetic receptor 1a) (79). Although it is known that, in ECs, ALK1 activates ECs through a SMAD1/5 pathway, whereas ALK5 inhibits EC activation through a SMAD2/3 pathway (80, 81), it is not known what role, if any, ALK3 plays in regulating EC phenotype. However, its upregulation by *Gax* implies that *Gax* may activate TGF-β signaling or render ECs more sensitive to TGF-β. Second, we noted the upregulation of three CDK inhibitors, $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF1/CIP1}$ (26, 61, 62), suggesting redundant mechanisms by which *Gax* can induce G_1 cell cycle arrest. Finally, we note that *Frizzled-2* was upregulated. Little is known about the potential role of *Frizzled* receptors and Wnt signaling in regulating postnatal angiogenesis, although *Frizzled-2* is known to be expressed in ECs and there is evidence suggesting Wnt signaling inhibits EC proliferation (82, 83). This data leads us to two potential other signaling pathways besides NF-κB to pursue in the future.

<u>Task 6</u>: ***Exploration of downstream pathways activating putative downstream targets of Gax identified by cDNA microarray. (Months 25-36.)***

a. **Northern and Western blots of genes identified in Task #6 in order to verify regulation by *Gax*. (Months 19-36.)**

Status: Complete.

Results and Discussion: We have now verified that a number of the genes identified in the cDNA microarray experiments as being downregulated by *Gax* are also downregulated. First, we examined several NF-κB-dependent genes, because that would represent independent verification that NF-κB signaling pathways are downregulated by *Gax* expression. We found that basal and TNF-α-induced expression of ICAM-1, VCAM-1, and E-selectin were all strongly inhibited by *Gax* expression (Figure 10). This is consistent with a role for *Gax* in inhibiting NF-κB-dependent gene expression. In addition, we noted that

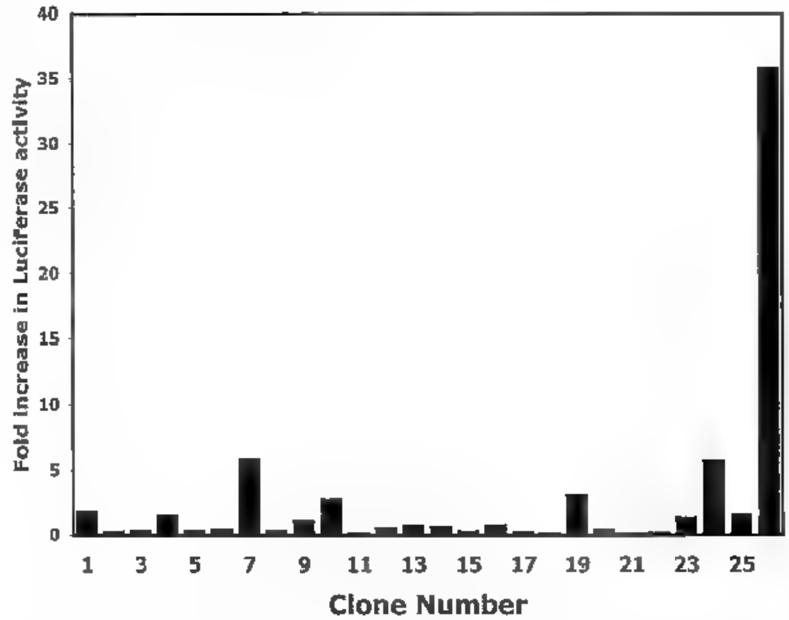
mRNAs for proangiogenic peptides such as VEGF and bFGF were also downregulated, at least at the message level (Figure 11). These observations are suggestive of a role for *Gax* in not only blocking NF-κB-dependent gene activity but for potentially blocking angiogenesis through inhibition of the autocrine stimulation of ECs.

b. Determination of the effect of *Gax* expression on sequence-specific DNA binding by NF-κB. (Months 24-36.)

Status: In progress.

Results and Discussion: Given that NF-κB activity has been implicated in the changes in phenotype and gene expression ECs undergo during angiogenesis caused by VEGF, TNF-α, and other factors, and that a number of NF-κB targets have been implicated in inducing angiogenesis (63, 77, 84-88), we wished to confirm the finding from cDNA microarray studies that *Gax* inhibits NF-κB activity in ECs. We therefore performed electrophoretic mobility shift assays utilizing nuclear extracts from HUVECs transduced with either Ad.r*Gax* or the control adenoviral vector Ad.GFP to measure binding to a probe containing an NF-κB consensus sequence (89). Specific binding to NF-κB consensus sequence by nuclear extracts from HUVECs transduced with Ad.*Gax* and then induced with TNF-α (10 ng/ml) was much reduced compared to that observed in controls (Figure 12), implying that *Gax* expression interferes with the binding of NF-κB to its consensus sequence. These new data imply that *Gax* also likely inhibits transcriptional activation by NF-κB. This suggests a method by which *Gax* may inhibit angiogenesis in breast cancer and an important hypothesis to test in the future.

A.



B.

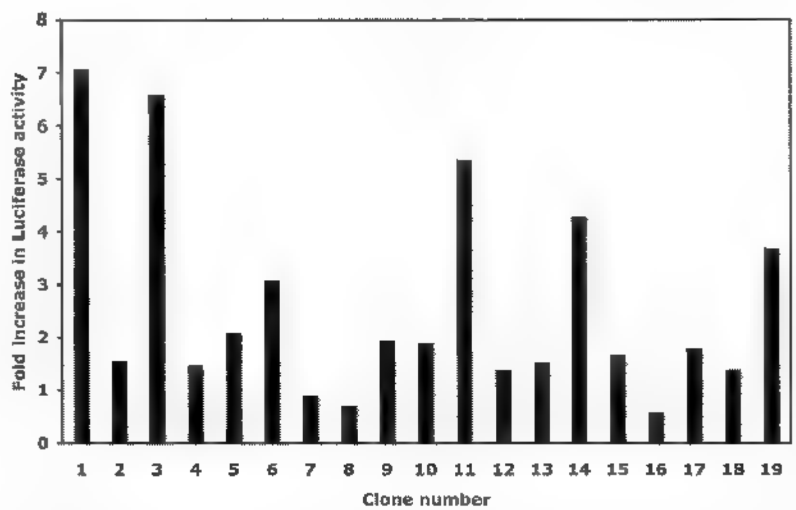


Figure 9. HMEC-1 constructs with inducible gene expression. A. Tetracycline-inducible (Tet-On) system. HMEC-1 cells were transduced with pTet-On, which introduces the rTA element. Cells were selected with Hygromycin B, and then Hygromycin B-resistant colonies selected and expanded. Cells from individual colonies were then transduced with pTRE-Luc, a plasmid in which Luciferase expression is driven by the Tet response element, which is active in the presence of tetracycline or doxycycline and silent otherwise. Luciferase expression was determined in the presence and absence of doxycycline Clone #26 showed the most induction with doxycyline. B Ecdysone-inducible system. HMEC-1 cells were transfected with the Ecdysone-inducible promoter and then transfected with the appropriate promoter-reporter construct in the presence and absence of Ponasterone A

TABLE I: GENES REGULATED BY *GAX* EXPRESSION

UPREGULATED GENES

Genbank no.	Gene	Function	Fold change	p
L37882	Frizzled homolog 2 (FZD2)	Signal transduction	30.4	<0.0001
NM_025151	Rab coupling protein (RCP)	Signal transduction	30.1	0.0026
AI678679	Bone morphogenetic protein receptor, type IA (BMPR1A, ALK3)	Signal transduction	27.9	0.0015
N74607	Aquaporin 3 (AQP3)	Transport	19.9	0.0011
AI983115	Class I cytokine receptor	Signal transduction	12.[illegible]	<0.0001
NM_002276	Keratin 19 (KRT19)	Structural protein	9.2	<0.0001
NM_004727	Solute carrier family 24 member 1 (SLC24A1)	Ion transport	9.2	0.0007
NM_004585	Retinoic acid receptor responder (tazarotene induced) 3	Cell growth inhibition	8.5	0.0077
K01228	Proalpha 1 (I) chain of type I procollagen	Structural protein	6.4	0.000[illegible]
NM_000361	*Thrombomodulin (THBD)*	*Coagulation*	*5.5*	*0.0006*
NM_006931	Solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3)	Biosynthesis/metabolism	5.3	0.0000
NM_000850	Glutathione S-transferase M4 (GSTM4)	Biosynthesis/metabolism	4.9	0.0009
NM_002064	Glutaredoxin (thioltransferase) (GLRX)	Biosynthesis/metabolism	4.9	0.0001
AF162769	Thioltransferase	Biosynthesis/metabolism	4.6	<0.0001
NM_002166	*Inhibitor of DNA binding 2 (ID2)*	*Transcriptional regulation*	*4.6*	*0.0001*
NM_017436	alpha1,4-galactosyltransferase, 4-N-acetylglucosaminyltransferase (A14GALT)	Biosynthesis/metabolism	4.3	0.0003
NM_005904	MAD (mothers against decapentaplegic) homolog 7 (MADH7)	Signal transduction	4.3	0.0006
NM_000170	Glycine dehydrogenase (GLDC)	Biosynthesis/metabolism	4.0	0.0003
NM_002222	Inositol 1,4,5-triphosphate receptor type 1 (ITPR1)	Signal transduction	4.0	0.0000
NM_000229	Lecithin-cholesterol acyltransferase (LCAT)	Biosynthesis/metabolism	4.0	0.0002
M25915	Complement cytolysis inhibitor (CLI)	Complement activation	3.7	<0.0001
AF326591	Fenestrated-endothelial linked structure protein (FELS)	Structural protein	3.7	<0.0001
NM_001666	Rho GTPase activating protein 4 (ARHGAP4)	Signal transduction	3.7	<0.0001
NM_006456	Sialyltransferase (STHM)	Biosynthesis/metabolism	3.7	0.0001
NM_000050	Argininosuccinate synthetase (ASS)	Biosynthesis/metabolism	3.7	<0.0001
AF035620	BRCA1-associated protein 2 (BRAP2)	Biosynthesis/metabolism	3.5	0.0002
M25915	Cytolysis inhibitor (CLI)	Complement activation	3.5	<0.0001
NM_006736	Heat shock protein, neuronal DNAJ-like 1 (HSJ1)	Stress response	3.5	0.0001
NM_000693	Aldehyde dehydrogenase 1 family, member A3 (ALDH1A3)	Biosynthesis/metabolism	3.5	<0.0001
NM_000213	*Integrin subunit, beta 4 (ITGB4)*	*Cell adhesion*	*3.5*	*0.0001*
NM_003043	Solute carrier family 6, member 6 (SLC6A6)	Transport	3.5	0.000[illegible]
AF010126	Breast cancer-specific protein 1 (BCSG1)	Unknown	3.2	0.0002
NM_005345	Heat shock 70kD protein 1A (HSPA1A)	Stress response	3.2	<0.0001
NM_006254	Protein kinase C, delta (PRKCD)	Signal transduction	3.0	0.0001
NM_000603	Nitric oxide synthase 3 (endothelial cell) (NOS3)	Biosynthesis/metabolism	3.0	<0.0001
U20498	Cyclin-dependent kinase inhibitor p19INK4D	Cell cycle	2.5	0.0004
NM_001147	*Angiopoietin 2 (ANGPT2)*	*Cell growth/chemotaxis*	*2.2*	*0.0023*
N33167	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	Cell cycle	2.1	0.0065

DOWNREGULATED GENES

Genbank no.	Gene	Function	Fold change	p
NM_002167	*Inhibitor of DNA binding 3 (ID3)*	*Transcriptional regulation*	*-2.0*	*0.0081*
D13889	*Inhibitor of DNA binding 1 (ID1)*	*Transcriptional regulation*	*-2.1*	*0.0052*
NM_001546	*Inhibitor of DNA binding 4 (ID4)*	*Transcriptional regulation*	*-2.1*	*0.0056*
M60278	***Heparin-binding epidermal growth factor-like growth factor***	***Cell growth/chemotaxis***	***-2.1***	***0.0056***
NM_001955	***Endothelin 1 (EDN1)***	***Cell growth/chemotaxis***	***-2.5***	***0.0007***
NM_000201	***Intercellular adhesion molecule 1 (ICAM1)***	***Signal transduction***	***-2.5***	***0.0059***
NM_004995	*Matrix metalloproteinase 14*	*Proteolysis*	*-2.7*	*0.0002*
NM_002006	*Fibroblast growth factor 2 (basic) (FGF2)*	*Cell growth/chemotaxis*	*2.8*	*0.0244*
NM_004428	*Ephrin-A1 (EFNA1)*	*Cell growth/chemotaxis*	*3.0*	*0.00[illegible]2*
AF021834	Tissue factor pathway inhibitor beta (TFPIbeta)	Coagulation	-3.0	0.0007
NM_016931	NADPH oxidase 4 (NOX4)	Biosynthesis/metabolism	-3.2	0.0029
NM_021106	Regulator of G-protein signalling 3 (RGS3)	Signal transduction	-3.5	0.0059
NM_002130	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 1 (soluble) (HMGCS1)	Biosynthesis/metabolism	3.5	0.0008
NM_001146	*Angiopoietin 1 (ANGPT1)*	*Cell growth/chemotaxis*	*-3.9*	*0.00[illegible]2*
NM_005658	***TNF receptor-associated factor 1***	***Signal transduction***	***-4.0***	***0.0086***
NM_001721	BMX non-receptor tyrosine kinase (BMX) mRNA	Signal transduction	-4.3	0.0007
NM_006226	Phospholipase C, epsilon (PLCE)	Signal transduction	4.3	0.0012
NM_006823	Protein kinase (cAMP-dependent, catalytic) inhibitor alpha (PKIA)	Signal transduction	-4.3	0.0002
NM_002425	*Matrix metalloproteinase 10*	*Proteolysis*	*4.4*	*0.0002*
NM_016315	CED-6 protein (CED-6)	Vesicle-mediated transport	-4.6	0.0059
NM_000600	***Interleukin 6 (interferon, beta 2) (IL6)***	***Cell growth/chemotaxis***	***-4.6***	***0.0020***
M68874	Phosphatidylcholine 2-acylhydrolase (cPLA2)	Signal transduction	-4.9	0.0007
U58111	***Vascular endothelial growth factor C (VEGF-C)***	***Cell growth/chemotaxis***	***-5.3***	***0.0020***
NM_003326	**Tumor necrosis factor (ligand) superfamily, member 4 (TNFSF4)**	**Signal transduction**	**-5.7**	**0.0021**
AB040875	Cystine-glutamate exchanger	Biosynthesis/metabolism	-6.1	0.0012
NM_006290	***Tumor necrosis factor-α-induced protein 3 (A20, TNFAIP3)***	***Apoptosis***	***-6.4***	***0.0009***
S69738	**Monocyte chemotactic protein human (MCP-1)**	**Cell growth/chemotaxis**	**-6.5**	**0.0303**
NM_012242	Dickkopf homolog 1 (DKK1)	Signal transduction	-8.0	0.0002
NM_002852	**Pentaxin-related gene, rapidly induced by IL-1 beta (PTX3)**	**Immune response**	**9.2**	**0.0142**
L07555	**Early activation antigen CD69**	**Signal transduction**	**10.6**	**0.0042**
NM_001078	***Vascular cell adhesion molecule 1 (VCAM1)***	***Cell adhesion***	***-13.0***	***0.0303***
NM_002993	**Granulocyte chemotactic protein 2**	**Cell growth/chemotaxis**	**-17.5**	**0.0059**
NM_012252	Transcription factor EC	Transcriptional regulation	18.5	0.0302
NM_000963	Prostaglandin-endoperoxide synthase 2	Biosynthesis/metabolism	-26.0	0.0303
NM_001993	***Coagulation factor III (thromboplastin, tissue factor)***	***Coagulation***	***-39.4***	***0.0022***
NM_000450	***E-selectin (SELE)***	***Cell adhesion***	***-62.6***	***0.0142***
M57731	***Chemokine (C-X-C motif) ligand 2 (CXCL2, GRO-beta)***	***Cell growth/chemotaxis***	***-79.6***	***0.0007***
NM_002090	***Chemokine (C-X-C motif) ligand 3 (CXCL3)***	***Cell growth/chemotaxis***	***-119.9***	***0.0029***
NM_000584	***Interleukin 8 (IL8)***	***Immune response***	***-181.3***	***0.0142***

NM_004591	*Chemokine (C-C motif) ligand 20 (CCL20)*	*Cell growth/chemotaxis*	*-237.6*	*0.0376*
NM_001511	*Melanoma growth stimulating activity, alpha/GRO-1/GRO-α (CXCL1)*	*Cell growth/chemotaxis*	*-238.9*	*0.0059*

Note: Boldface=genes induced by NF-κB activity; italicized=genes involved in regulating angiogenesis

c. Determination of the effect of *Gax* expression on transactivation by NF-κB. (Months 24-36.)

Status: Complete.

Results and Discussion: We examined other aspects of the NF-κB signaling cascade to determine at what level *Gax* inhibits it. First, we studied the effect of *Gax* expression on an NF-κB-dependent promoter

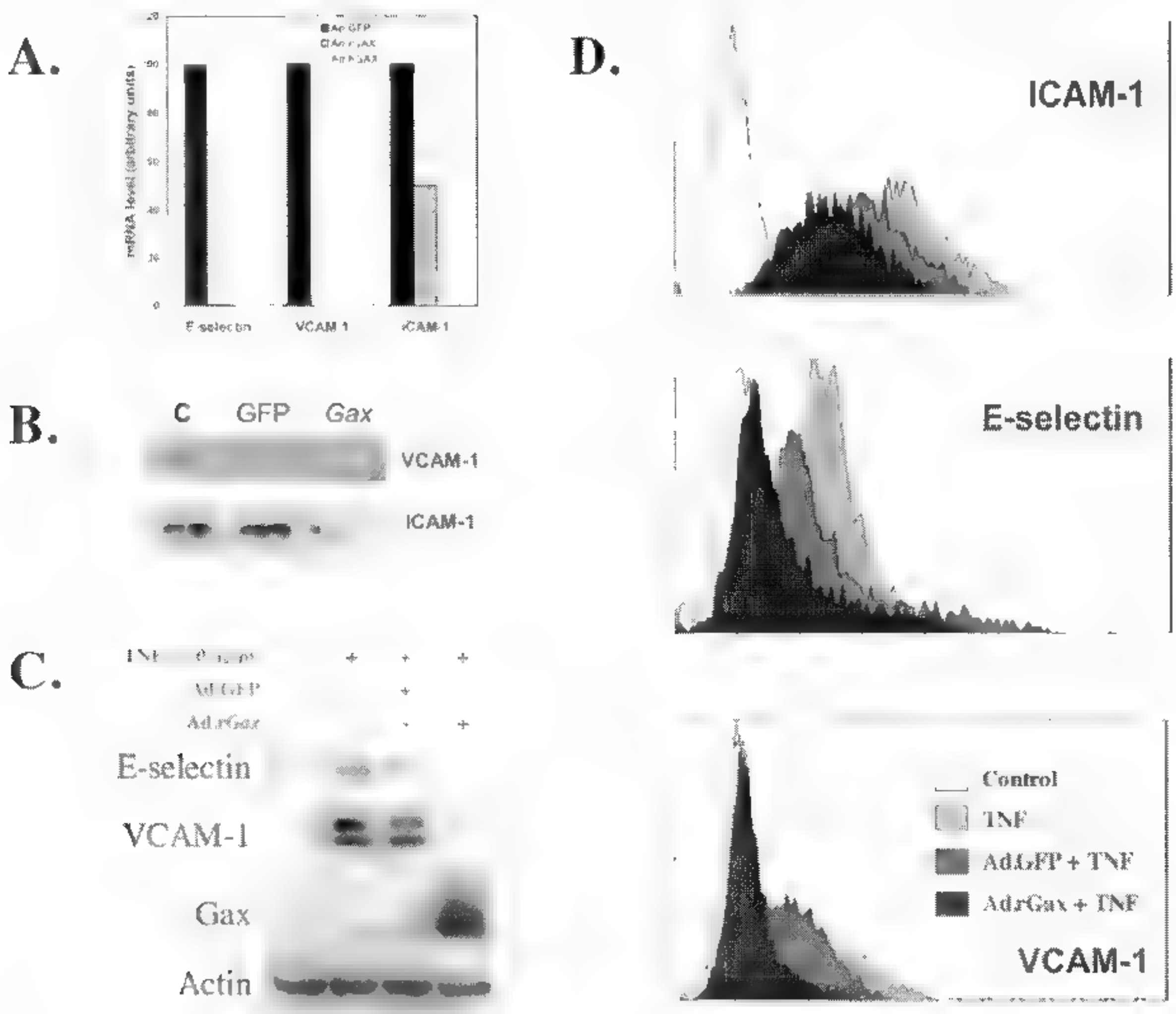


Figure 10. Effect of *Gax* expression on the level of E-selectin, VCAM-1, and ICAM-1. A. Quantitative real time PCR. Cells were harvested for total RNA isolation. Total RNA was then subjected to quantitative real time RT-PCR using TaqMan primers and probes specific for each gene and the results normalized to GAPDH. Units were chosen such that controls were set to 100. A very strong downregulation of E-selectin, VCAM-1, and ICAM-1 message level was observed. **B. *Gax* downregulates VCAM-1 and ICAM-1 proteins.** HUVECs were transduced with Ad.*rGax* or Ad.GFP and then incubated overnight. Cells were harvested for total protein and 50 μg protein was subjected to Western blot with appropriate antibodies (C= control with no virus, GFP=Ad.GFP; *Gax*=Ad.*rGax*). E-selectin could not be visualized in unstimulated HUVECs. **C. *Gax* blocks upregulation of VCAM-1 and E-selectin.** HUVECs were transduced with Ad.*rGax* or Ad.GFP and then incubated overnight, after which they were stimulated with 10 ng/ml TNF-α for one hour. Cells were harvested for total protein and 50 μg protein was subjected to Western blot with appropriate antibodies. Expression of Gax from the adenoviral vector was verified by Western blot with antibodies against Gax previously described. **D. *Gax* downregulates cell surface expression of ICAM-1, E-selectin, and ICAM-1.** HUVECs transduced overnight with either Ad.GFP or Ad.*rGax* at an MOI 100 were stimulated with TNF-α 10 ng/ml for 4 hours and then harvested for flow cytometry using appropriate antibodies. Ad. blocked the expression of VCAM-1, E-selectin, and ICAM-1

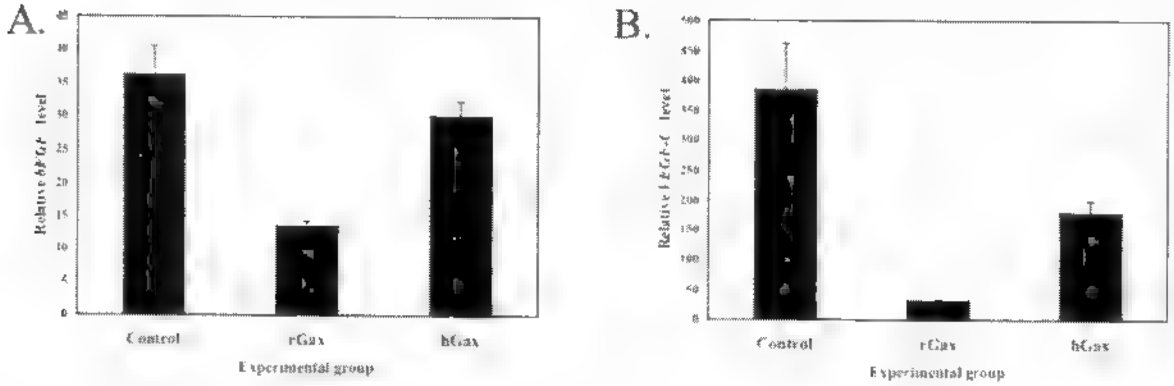


Figure 11. ***Gax*** **downregulates proangiogenic factors expressed by ECs.** HUVECs were transduced with either Ad.GFP (control), Ad.r*Gax*, or Ad.h*Gax* at MOI 100. After 24 hrs., cells were harvested for total RNA, which was then subjected to real time quantitative RT-PCR as described (Specific Aim 1) VEGF-C and bFGF message levels were normalized to GAPDH message Units are arbitrary. **A.** bFGF **B.** VEGF-C.

activity. Using an IL-6 promoter-Luciferase construct (90), we performed cotransfection experiments using a *Gax* expression vector (pCGN-*Gax*) and a vector expressing a truncated version of *Gax* lacking the homeodomain (pCGN-*Gax*ΔHD and measured the effect of *Gax* expression in IL-6 promoter activity. *Gax* inhibited IL-6 promoter activity in a dose-dependent fashion, an effect that was only marginally affected by deleting the homeodomain (Figure 13). This implies that the mechanism by which *Gax* blocks NF-κB-dependent gene expression is likely not a direct competition between *Gax* and the NF-κB complex for DNA binding on the IL-6 promoter, given that the homeodomain is the DNA-binding domain of *Gax* (26). Although these results are very preliminary, they imply that *Gax* may actually inhibit NF-κB signaling upstream of NF-κB-dependent promoters.

d. Determination of the effect of *Gax* expression on NF-κB expression and signaling. (Months 24-36.)

Status: In progress.

Results and Discussion: With the help of our collaborator, Dr. Arnold Rabson (UMDNJ-Robert Wood Johnson Medical School, Piscataway, NJ), we have begun to measure NF-κB and IκB expression by Western blot and to measure NF-κB signaling by measuring IKK activity and IκB activity. We first looked at the effect of *Gax* expression on IκBα and IκBβ degradation in response to TNF-α stimulation. HMEC-1 cells were stimulated with 10 ng/ml TNF-α, and Western blots performed at different time courses. Our preliminary results suggest that *Gax* might inhibit the signal-induced degradation of IκBα but not IκBβ (Figure 14), although subsequent experiments have cast doubt on this initial result (data not shown). Further experiments are in progress to determine which is the correct result. We will also determine the effects of *Gax* expression on the nuclear translocation of the NF-κB complex in response to stimulation by factors known to induce NF-κB activity in ECs, including VEGF and TNF-α. We have recently begun these experiments.

KEY RESEARCH ACCOMPLISHMENTS

Our key research accomplishments over the course of this award include:

1. Demonstrated that mitogens and proangiogenic factors regulate *Gax* expression in ECs in a manner similar to that observed in vascular smooth muscle cells, with its expression maximal in quiescent cells and rapidly downregulated after ECs are treated with mitogens, VEGF, or bFGF.
2. Demonstrated that proangiogenic factors secreted by breast cancer cells downregulate *Gax* expression in ECs.

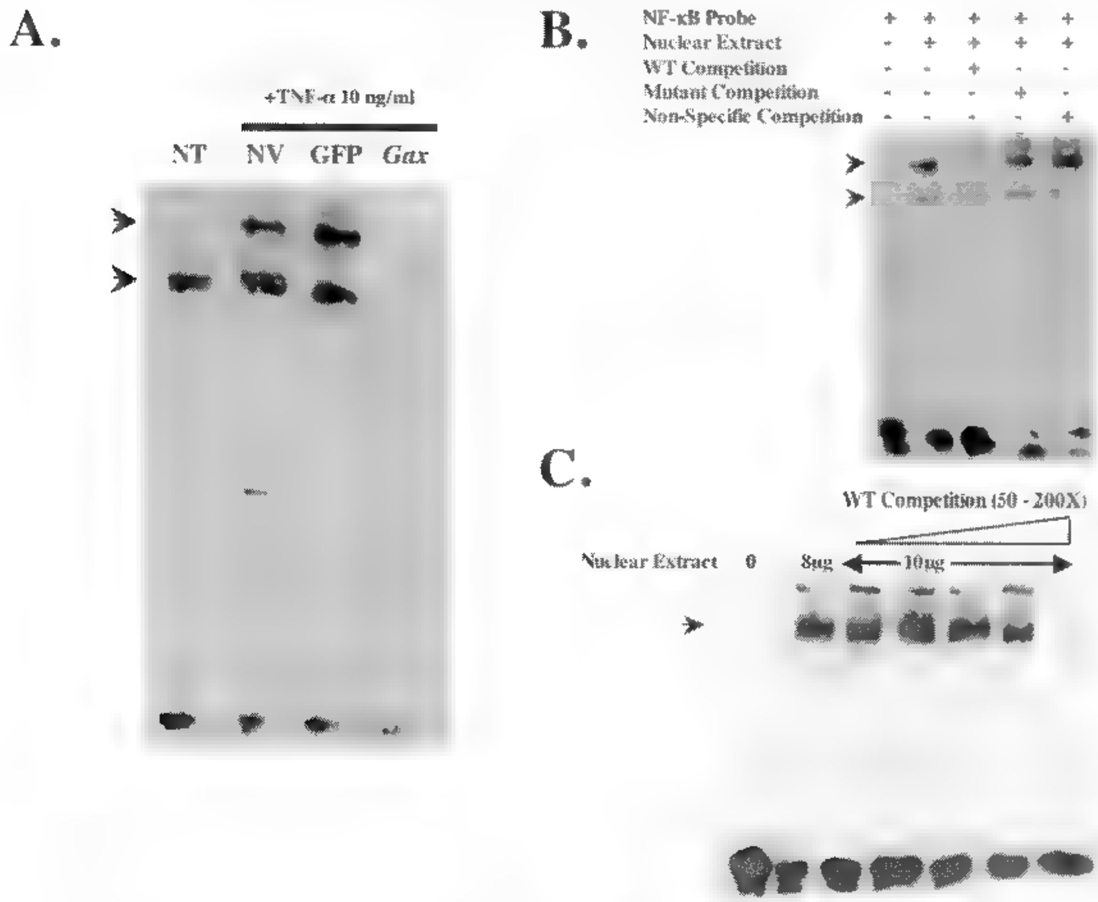


Figure 12. ***Gax*** **expression inhibits NF-κB binding to its consensus sequence. A. Gax blocks NF-κB binding to its consensus sequence.** HUVECs were infected with adenovirus containing GFP or r*Gax*, incubated overnight in EGM-2, and then induced with 10 ng/ml TNF-α for 1 hour. Controls were not induced with TNF-α. Nuclear extracts were prepared with the NE-PER nuclear extraction reagent (Pierce) Nuclear extracts were incubated with biotinylated oligonucleotides, containing the consensus NF-κB binding site, and the reactions were electrophoresed on a 6% acrylamide gel. The reactions were transferred to positively charged nylon membrane and detected with the LightShift EMSA kit (Pierce) Arrows denote NF-κB specific bands, and bands at the bottom of the gels represent unbound probe. **B and C. Control EMSAs.** These demonstrate failure of a random sequence oligonucleotide and an NF-κB consensus sequence with a point mutation that abolishes DNA binding to compete with wild-type NF-κB sequence **(B)** and competition with an excess of unlabeled wild-type NF-κB oligonucleotide **(C)**. **Legend:** NT no treatment; NV=no virus

3. Completed analysis of initial cDNA microarray data obtained near the end of Year One and showed that *Gax* downregulates the expression of NF-κB-dependent genes.
4. Confirmed cDNA microarray results for several genes identified in our initial cDNA microarray experiment at the message and protein level.
5. Demonstrated that *Gax* expression inhibits EC migration towards serum and proangiogenic stimuli.
6. Determined that *Gax* expression inhibits angiogenesis *in vivo* in the Matrigel plug assay.
7. Determined that Gax expression downregulates the expression of proangiogenic factors in ECs.
8. Determined that *Gax* expression inhibits activation of NF-κB-dependent promoters.
9. Ruled out an interaction between *Gax* and IκBα as a mechanism of *Gax* inhibition of NF-κB signaling.

10. Begun to examine whether there is an interaction between *Gax* and IκBα or IκBβ as a mechanism of *Gax* inhibition of NF-κB signaling.
11. Demonstrated that *Gax* expression inhibits phosphorylation of ERK1/2 (data not shown).
12. Determined that *Gax* expression inhibits activation of NF-κB-dependent promoters.

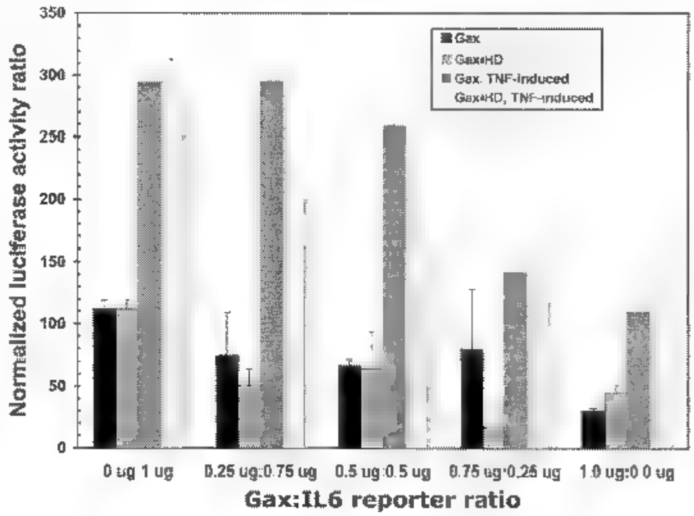


Figure 13. *Gax* expression inhibits NF-κB-dependent promoter activity. HUVECs were co-transfected with an IL-6 promoter construct plus either a vector expressing *Gax* (pCGN-*Gax*) or *Gax* lacking its homedomain (pCGN-*Gax*ΔHD) and then stimulated with TNF-α for four hours. Cells were harvested for Luciferase activity and normalized to *Renilla* Luciferase, which had been included to control for transfection efficiency. *Gax* inhibits IL-6 promoter activity, an effect that does not depend upon its homeodomain.

REPORTABLE OUTCOMES

Journal articles:

1. **Gorski DH** and AD Leal (2003). Inhibition of endothelial cell activation by the homeobox gene *Gax*. *J. Surg. Res.* **111**: 91-99.
2. **Gorski DH**, and K Walsh (2003). Control of vascular cell differentiation by homeobox transcription factors. *Trends Cardiovasc Med* **13**: 213-220.
3. Patel, S., Leal, A D., and **D. H. Gorski** (2005). The homeobox gene *Gax* inhibits angiogenesis through inhibition of NF-κB-dependent endothelial cell gene expression. *Cancer Res.* **65**:1414-1424.

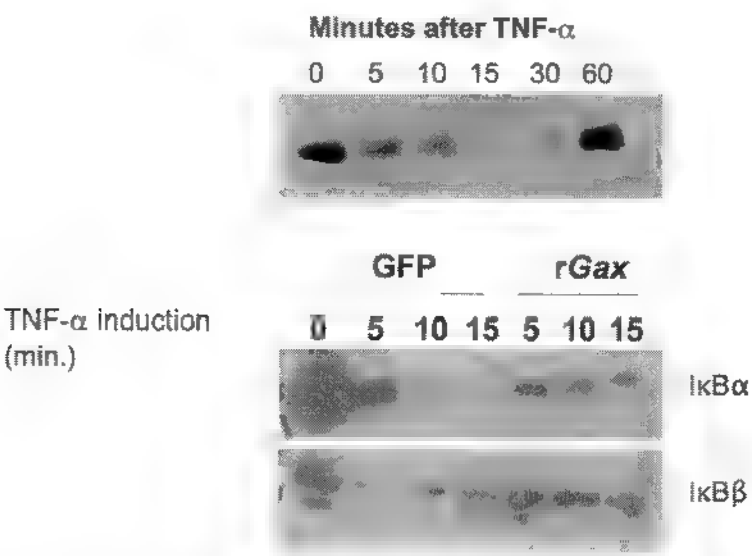


Figure 14. *Gax* expression inhibits IκBα degradation. Serum-starved HUVECs were treated with control vector (Ad.GFP) or Ad.r*Gax* at MOI=100, stimulated with TNF-α, and then harvested for Western blot with antibodies to IκBα and IκBβ. Gax expression blocks the degradation of IκBα but not IκBβ

Abstracts

1. **Gorski, D. H.** (2002) The homeobox gene *Gax* induces p21 expression and inhibits vascular endothelial cell activation. *Ann. Surg. Oncol.* 9:S42
2. Patel, S., and **D. H. Gorski** (2004). Inhibition of endothelial cell activation and angiogenesis by the homeobox gene *Gax* is associated with downregulation of nuclear factor-κB (NF-κB)-dependent gene expression. *Proc. Amer. Assoc. Cancer Res.*45:77. Presented at the Annual Meeting of the American Association for Cancer Research, Orlando, FL, March 28, 2004

Scientific presentations at national meetings:

1. **Gorski, D. H.** *The homeobox gene* **Gax** *induces p21 expression and inhibits vascular endothelial cell activation* The Society of Surgical Oncology Meeting, Denver, CO, March 14-17, 2002
2. Patel, S., A. Leal, and **D. H. Gorski** (2005). *Inhibition of endothelial cell activation and angiogenesis by the homeobox gene Gax is associated with downregulation of nuclear factor κB (NF-κB)-dependent gene expression.* Plenary Session, Society of Surgical Oncology Meeting, Atlanta, GA, March 3-6, 2005.

Funding obtained based on work done on this project:

1 R01 CA111344-01 **National Cancer Institute** **Role: Principal investigator**	6/1/2005 – 5/31/2010	30% effort

Mechanism of angiogenesis inhibition by a homeobox gene

The overall goal of this project is to define more clearly the mechanism by which *Gax* inhibits endothelial cell activation and angiogenesis, specifically how it does so *in vivo* and how it inhibits NF-κB-dependent gene activation. A significant portion of the preliminary data used to support this grant application was obtained with the generous support of the present U. S. Army Career Development Award

CONCLUSIONS

Homeobox genes are master regulatory genes with diverse functions in many cell types, both during embryogenesis and in the adult (1, 3, 4, 6, 91). It is therefore not surprising that recently they have been implicated as important transcriptional regulators controlling endothelial cell phenotype during tumor-induced angiogenesis (7, 8, 10, 12, 50, 92). Until recently, little was known about how homeobox genes might influence endothelial cell phenotype and behavior during breast cancer-induced angiogenesis. However, evidence for their involvement in the phenotypic changes endothelial cells undergo during angiogenesis is now accumulating. For instance, Patel *et al* reported an endothelial cell-specific variant of *HOXA9* whose expression is regulated by tumor necrosis factor-α, which is proangiogenic (93). More direct evidence for the importance of homeobox genes in angiogenesis exists for *HOXD3* (7). *In vivo*, sustained expression of *HOXD3* on the chick chorioallantoic membrane (CAM) retains endothelial cells in an invasive state and prevents vessel maturation, leading to vascular malformations and endotheliomas. In diabetic mice, *HOXD3* expression is impaired in endothelial cells, as is its upregulation after wounding (50). Moreover, *HOXD3* expression is elevated in breast cancer tumor vasculature as compared to normal vasculature, as measured by *in situ* hybridization (13). More recently, overexpression of another homeobox gene, *HOXB3* has been shown to result in an increase in capillary vascular density and angiogenesis, and its blockade by antisense results in impaired capillary morphogenesis (8). Similarly, *HOXB5* contributes to the development and differentiation of flk-1-positive angioblasts (11). In contrast, *HOXD10* inhibits EC conversion to the angiogenic phenotype, and sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis *in vivo* (94). Consistent with this, *HOXD10* expression is decreased in breast cancer vasculature (12). Another homeobox gene, *Hex*, has a more complex role, being upregulated in angiogenic vasculature (92, 95, 96) during embryogenesis but inhibiting angiogenesis *in vitro* and *in vivo* (9, 10). Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating batteries of downstream genes to induce or inhibit the phenotypic changes in endothelial cells associated with angiogenesis. These observations are what initially led us to look for additional homeobox genes likely to be involved in the final transcriptional control of genes determining angiogenic phenotype in breast cancer. Because blocking aberrant angiogenesis has the potential to be an effective strategy to treat or prevent multiple diseases, understanding how downstream transcription factors

integrate upstream signals from pro- and anti-angiogenic factors to alter global gene expression and produce the activated, angiogenic phenotype, will be increasingly important in developing effective antiangiogenic therapies for breast cancer.

Based on our data, we postulated that at least one additional homeobox gene, *Gax,* is also likely to have an important role in the phenotypic changes that occur in ECs during angiogenesis and therefore wanted to study its role in regulating breast cancer-induced angiogenesis. We examined *Gax* expression in vascular ECs. We found that *Gax* is expressed in this cell type and that it has many of the same activities as in VSMCs. In addition, its expression inhibited EC tube formation on Matrigel *in vivo* (19). These observations led us to the present study, in which we wished to elucidate further the role(s) *Gax* may have in regulating angiogenesis, in particular breast cancer-induced angiogenesis. Consistent with its regulation in VSMCs, in ECs, *Gax* is rapidly downregulated by serum, proangiogenic, and pro-inflammatory factors (Figures 2 and 3), and is able to inhibit EC migration *in vitro* (Figure 7) and angiogenesis *in vivo* (Figure 8) These observations led us to examine the mechanism by which *Gax* inhibits EC activation utilizing cDNA microarrays to examine global changes in gene expression due to *Gax*. In addition to observing that *Gax* upregulates cyclin kinase inhibitors and downregulates a number of proangiogenic factors, we also found that *Gax* inhibits the expression of a number of NF-κB target genes (Table 1). Consistent with the cDNA microarray data, *Gax* inhibits the binding of NF-κB to its consensus sequence (Figure 12).

The NF-κB/Rel proteins are an important class of transcriptional regulators that play a central role in modulating the immune response and promoting inflammation and cancer by regulating the expression of genes involved in cell growth, differentiation, and apoptosis. In many cell types, NF-κB promotes cell survival in response to pro-apoptotic stimuli, induces cellular proliferation, or alters cell differentiation. Several lines of evidence have implicated NF-κB activity in regulating EC phenotype during inflammation and angiogenesis and, in particular, the classic activation of RelA-containing heterodimers (63, 73, 77, 84-87, 97). For example, proangiogenic factors such as VEGF (73), TNF-α (97), and platelet-activating factor (77) can all activate NF-κB signaling and activity in ECs. In addition, inhibition of NF-κB activity inhibits EC tube formation *in vitro* on Matrigel (87, 98), and pharmacologic inhibition of NF-κB activity suppresses retinal neovascularization *in vivo* in mice. (99) Moreover, ligation of EC integrin $\alpha_V\beta_3$ by osteopontin protects ECs against apoptosis induced by serum withdrawal, an effect that is due to NF-κB-dependent expression of osteoprotogerin (85). Similarly, $\alpha_5\beta_1$-mediated adhesion to fibronectin also activates NF-κB signaling and is important for angiogenesis, and inhibition of NF-κB signaling inhibits bFGF-induced angiogenesis (63). One potential mechanism by which NF-κB signaling may promote angiogenesis is through an autocrine effect, whereby activation of NF-κB induces expression of proangiogenic factors such as VEGF, as has been reported for platelet-activating factor-induced angiogenesis (77). Alternatively, the involvement of NF-κB in activating EC survival pathways is also likely to be important for sustaining angiogenesis (98).

Although NF-κB activity can influence the expression of homeobox genes (93, 100), there have been relatively few reports of functional interactions between homeodomain-containing proteins and NF-κB proteins. The first such interaction reported was between IκBα and HOXB7, where IκBα was found to bind through its ankyrin repeats to the HOXB7 protein and potentiate HOXB7-dependent gene expression (101). More recently, it was reported that IκBα can also potentiate the activity of other homeobox genes, including *Pit-1* and *Pax-8*, through the sequestration of specific histone deacetylases (102). In contrast, Oct-1 can compete with NF-κB for binding to a specific binding site in the TNF-α promoter (103). In addition, at least one interaction has been described in which a homeobox gene directly inhibits NF-κB-dependent gene expression, an interaction in which Cdx2 blocks activation of the COX-2 promoter by binding p65/RelA (104). It remains to be elucidated whether *Gax* inhibits NF-κB-dependent gene expression by a similar mechanism or a different one. Regardless of the mechanism, however, our observations made while doing

the research funded by this Idea Award, to our knowledge, represent the first description of a homeobox gene that not only inhibits phenotypic changes that occur in ECs in response to proangiogenic factors, but also inhibits NF-κB-dependent gene expression in vascular ECs. These properties suggest *Gax* as a potential target for the antiangiogenic therapy of breast cancer. In addition, understanding the actions of *Gax* on downstream target genes, signals that activate or repress *Gax* expression, and how *Gax* regulates NF-κB activity in ECs is likely to lead to a better understanding of the mechanisms of breast cancer-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of breast cancer. It is these aims that we will continue to pursue utilizing the new support of our R01 grant, which could not have been obtained were it not for the preliminary data we developed with the support of the Army. In addition, our experiments have implicated *Gax* activity in possibly modulating Wnt and TGF-β signaling in breast cancer ECs, suggesting two other broad areas of research for understanding *Gax* function in regulating breast cancer angiogenesis.

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APPENDICES

Publications during period of report:

1. **Gorski DH** and AD Leal (2003). Inhibition of endothelial cell activation by the homeobox gene *Gax*. *J. Surg. Res.* **111**: 91-99.

2. **Gorski DH** and K Walsh (2003). Control of vascular cell differentiation by homeobox transcription factors. *Trends Cardiovasc Med* **13**: 213-220.

3. Patel, S., Leal, A. D., and **D. H. Gorski** (2005). The homeobox gene *Gax* inhibits angiogenesis through inhibition of NF-κB-dependent endothelial cell gene expression. *Cancer Res.* **65**:1414-1424.

APPENDIX 1

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Inhibition of Endothelial Cell Activation by the Homeobox Gene *Gax*

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Background. **Angiogenesis is critical to tumor growth. *Gax*, a homeobox transcription factor whose expression in the adult is restricted mainly to the cardiovascular system, strongly inhibits growth factor-stimulated phenotypic modulation of vascular smooth muscle cells *in vitro* and *in vivo*. The function of *Gax* in vascular endothelium is unknown, but we hypothesized that it may play a similar role there. We therefore studied *Gax* expression in vascular endothelial cells and its effects on proliferation and tube formation.**

Materials and methods. ***Gax*** **expression in normal endothelial cells was examined *in vitro* by Northern blot and reverse transcriptase polymerase chain reaction and *in vivo* by immunohistochemistry. A replication-deficient adenovirus was then used to express *Gax* in human umbilical vein endothelial cells (HUVECs). HUVEC proliferation, ^{3}H-thymidine uptake, p21 expression, and tube formation on reconstituted basement membrane were measured at different viral multiplicities of infection.**

Results. ***Gax*** **mRNA was detected in HUVECs by reverse transcriptase polymerase chain reaction and Northern blot analysis and in normal vascular endothelium by immunohistochemistry. Compared with controls transduced with a virus expressing β-galactosidase, *Gax* strongly inhibited HUVEC proliferation and mitogen-stimulated ^{3}H-thymidine uptake. p21 expression in HUVECs transduced with *Gax* was increased up to 5-fold as measured by Northern blot, and p21 promoter activity was activated by 4- to 5-fold. Tube formation on Matrigel was strongly inhibited by *Gax* expression.**

Conclusions. ***Gax*** **is expressed in vascular endothelium and strongly inhibits endothelial cell activation in response to growth factors and tube formation *in vitro*. These observations suggest that *Gax* inhibits endothelial cell transition to the angiogenic phenotype in response to proangiogenic growth factors and, as a negative regulator of angiogenesis, may represent a target for the antiangiogenic therapy of cancer.**

Key Words: **angiogenesis; homeobox genes; transcription factors; vascular endothelium.**

INTRODUCTION

Vascular remodeling plays a critical role in the biology of tumors, whose growth without a blood supply is limited to less than 1 mm in diameter by diffusion of oxygen and nutrients through the interstitial fluids [1]. To overcome this limitation, tumors secrete proangiogenic factors, such as vascular endothelial growth factor (VEGF) [2] and basic fibroblast growth factor (bFGF) [3], to stimulate the ingrowth of new blood vessels [1, 4]. To form new tumor vasculature, endothelial cells undergo profound phenotypic changes, many of which are similar to the phenotypic changes tumor cells undergo when invading the surrounding stroma [1, 5, 6]. They degrade their basement membrane and invade the surrounding tissue, migrate towards the proangiogenic stimulus secreted by the tumor, and then form tubular structures and finally neovasculature [1, 7]. Although the receptors and signaling pathways activated by proangiogenic factors and cytokines have been extensively studied in endothelial cells [8, 9], much less is known about the molecular biology of the downstream transcription factors that regulate the tissue-specific gene expression controlling endothelial cell growth and differentiation and are activated by these signaling pathways. These transcription factors represent a common mechanism that can be influenced by the interaction of multiple signal-

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ing pathways and therefore might represent targets for the antiangiogenic therapy of cancer.

To understand the transcriptional control of tumor-induced angiogenesis and thereby potentially identify new ways to target it therapeutically, we decided to study the role of homeobox transcription factors in regulating the phenotypic changes that occur in endothelial cells when stimulated with proangiogenic factors. Because of their ubiquitous role as regulators of cell proliferation, migration, and differentiation, as well as body plan formation and organogenesis during embryogenesis in vertebrates and invertebrates [10, 11] and as oncogenes and tumor suppressors in various human cancers [12, 13], of all the various classes of transcription factors, we considered homeobox genes as especially likely to be important in regulating endothelial cell phenotype during angiogenesis.

Among homeobox genes, *Gax* (*G*rowth *A*rrest-specific homeobo*X*) has several characteristics that suggest it as a candidate for a role as an inhibitor of the endothelial cell phenotypic changes that occur as a result of stimulation by proangiogenic factors. Originally isolated from vascular smooth muscle [14], in the adult *Gax* expression is largely restricted to the cardiovascular system [14, 15]. In vascular smooth muscle cells, *Gax* expression is downregulated by mitogens [14, 16] and upregulated by growth arrest signals [14, 17]. Consistent with this observation, *Gax* expression induces G_1 cell cycle arrest [18] and inhibits vascular smooth muscle cell migration, downregulating the expression of integrins, $\alpha_V\beta_3$ and $\alpha_v\beta_5$ [19], both of which are associated with the synthetic state in vascular smooth muscle cells and the angiogenic phenotype in endothelial cells [19, 20]. *In vivo*, *Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury [21]. Because *Gax* expression is largely confined to the cardiovascular system and mesoderm-derived structures [15, 22], we considered it likely that *Gax* is also expressed in endothelial cells because endothelial cells are also derived from mesoderm. Because of its activities in vascular smooth muscle cells, we further hypothesized that *Gax* may be involved in inhibiting the phenotypic changes that occur in endothelial cells in response to stimulation with proangiogenic factors. In this report, we show that *Gax* is also expressed in vascular endothelial cells and inhibits endothelial cell cycle activation and tube formation in response to proangiogenic factors, suggesting that it has a role as a negative regulator of angiogenesis

MATERIALS AND METHODS

Cells and Cell Culture

Human umbilical vein endothelial cells were obtained from Cambrex Biosciences (Walkersville, MD) and cultured as previously described [23] according to manufacturer's instructions in EGM 2 medium (Cambrex Biosciences, Walkersville, MD) For experiments, recombinant $VEGF_{165}$ (R & D Systems, Minneapolis, MN) was substituted in the media at the concentrations indicated for the proprietary VEGF solution

Plasmid and Adenoviral Constructs

The *Gax* cDNA was maintained in pBluescript SK+ vectors and excised as needed for use as probes for Northern blots. Adenoviral constructs expressing the human and rat homologs of *Gax* (Ad *hGax* and Ad *rGax*, respectively) conjugated to the α-hemagluttinin (HA) epitope were a kind gift of Dr. Kenneth Walsh (Boston University, Boston, MA) [18], as was the control adenoviral vector expressing β galactosidase (Ad.β *Gal*) Both human and rat isoforms of Gax were used to verify that both isoforms have similar activity. The control adenoviral vector expressing green fluorescent protein (Ad.*GFP*) was a kind gift of Dr. Daniel Medina (The Cancer Institute of New Jersey, New Brunswick, N J). Viral titers were determined by plaque assay Prior to the use of Ad.*hGax* or Ad *rGax* in HUVECs, expression of *Gax* mRNA and protein in cells transduced with these adenoviral constructs were verified by Northern and Western blot (not shown). The p21 cDNA and p21 promoter constructs were also obtained from Dr. Kenneth Walsh and are the same constructs used in other studies [18] The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) cDNA used as a probe for Northern blots was the same construct used in another study [14]

Immunohistochemistry

Tissue sections were obtained from human surgical specimens and fixed and imbedded in paraffin according to standard procedures, with sections dehydrated through xylenes and then rehydrated through graded ethanols [15]. Staining with a polyclonal rabbit anti-Gax antibody, which labels rat, human, and mouse Gax protein, was performed according to previously described methods, except that the dilution used was 1,1000 [15]. A biotin labeled goat anti-rabbit IgG (Sigma Corporation, St. Louis, MO) was used as a secondary antibody, and Gax staining was visualized using Vectastain ABC (Vector Laboratories, Burlingame, CA). Background staining was assessed by staining sections without primary antibody. All tissue specimens were obtained from a protocol approved by the Institutional Review Board of the University that protects the privacy of the patients from which the samples were obtained

Northern Blots

Northern blots measuring *Gax* expression were performed as previously described [14]. Briefly, total RNA (30 μg) was isolated from cultured cells using the guanidinium thiocyanate method [24] subjected to electrophoresis through formaldehyde-containing agarose gels, capillary blotted to nylon membranes using 10× SSC as the transfer buffer, fixed to the membrane using ultraviolet crosslinking, and then hybridized to the *Gax* cDNA labeled with ^{32}P by random priming in Church buffer [25]. Blots were exposed to Kodak XAR 5 X-ray film with an intensifying screen at −80° C Blots were then stripped with 0 1× SSC plus 0.1% SDS at 95°C and reprobed with the GAPDH cDNA to verify equal RNA loading. Hybridization temperatures were 55°C for *Gax*, p21, and GAPDH probes, and all blots were washed to a stringency of 0 2× SSC at 65°C. For p21 Northern blots, autoradiographs were scanned and band intensities determined with NIH Image v.1.6 p21 message levels were then normalized to GAPDH levels, and the fold induction of p21 determined

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RNA was isolated as described above from HUVECs and used in RT PCR to detect *Gax* transcripts. Total RNA (5 μg) was subjected to

reverse transcriptase reaction with MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) using random hexamers (Invitrogen, Carlsbad, CA) Because *Gax* has a single exon [26], all samples were treated with RNAse-free DNAse I (Ambion, Austin, TX) before being subjected to reverse transcription. As a further means of verifying that there was no genomic DNA contamination, control reactions with no reverse transcriptase were also subjected to PCR. To check the integrity of the RNA, the same reverse transcriptase reactions used to detect *Gax* were subjected to PCR using β actin–specific primers. Human *Gax* primer sequences were: 5'-GTCAGAAGT-CAACAGCAAACCCAG-3', sense; 5' CACATTCACCAGTTCCTTTT CCCGAGCC 3', antisense; product size 247 bp, from nucleotides 566 to 812 (26). Human β-actin primer sequences were: 5'-ATCCG-CAAAGACCTGT-3', β-actin sense; and 5' GTCCGCCTAGAAGC AT-3' β-actin antisense; product size 270 bp, from nucleotides 906 to 1175 [27] Before *Gax* primers were synthesized, their sequences were subjected to a BLAST [28] search against the Genbank database to detect any possibility that they might bind to or amplify genes other than *Gax*. Before running assays on experimental samples, each primer set, annealing conditions, Mg^{2} concentration, and primer and probe concentration were optimized using plasmids containing the cDNA of interest Reaction mixtures (25 μl) were used containing 0.75 U *Taq* polymerase (Gibco BRL), reaction buffer, 0 2 mM dNTPs, plus the optimized concentrations of $MgCl_2$, probe, and primers for each primer set The PCR cycle consisted of an initial 5 min denaturation step at 95°C, followed by 35 cycles of denaturation at 95°C for 30 s, annealing at 56°C (*Gax*) or 54°C (β actin) for each primer for 60 s, and extension at 72°C for 60 s.

Cell Proliferation and ^{3}H-Thymidine Incorporation

The effect of *Gax* overexpression on mitogen stimulated ^{3}H thymidine incorporation was examined in HUVECs. For cell proliferation, randomly cycling HUVECs in 6-well plates (20,000 cells/plate) were transduced for 12 h with Ad *Gax* or Ad.β-*gal* at varying MOIs, after which they were washed 3 times with phosphate buffered saline and then placed in fresh medium EGM 2 supplemented with 10 ng/ml VEGF$_{165}$). After infection, every day 3 wells for each experimental group were trypsinized and viable cells counted with cell viability determined by Trypan blue exclusion. For ^{3}H thymidine uptake studies, HUVECs were made quiescent by serum starvation for 24 h in medium containing 0.1% fetal bovine serum (FBS) at which point the cells were transduced with Ad.*Gax* or Ad β*Gal* and incubated in 0.1% FBS for an additional 24 h. The cells were then stimulated with medium containing 10% FBS and 10 ng/ml VEGF$_{165}$ for 24 h in the presence of 0 2 μCi/ml ^{3}H thymidine (Amersham, Piscataway, NJ), after which trichloroacetic acid precipitable counts were measured

Transactivation of the p21 Promoter

Subconfluent HUVECs were plated in 6-well plates and allowed to attach for 4 h. They were then infected with different MOIs of Ad.*hGax*, Ad.*r*Gax, or Ad *GFP* overnight, then transfected with p21 promoter Luciferase reporter construct. Transfection was performed using 2 μg p21 Luciferase plasmid per well, plus 0 2 μg pRL SV (Promega, Madison, WI), which contains the cDNA for *Renilla reniformis* Luciferase downstream from the SV40 promoter as its reporter instead of the cDNA for firefly Luciferase, as a control for transfection efficiency. Firefly and *Renilla* Luciferase activities were measured using the Dual Luciferase Assay Kit (Promega, Madison, WI), and the firefly Luciferase activity from the p21-Luciferase promoter construct normalized to the constitutive *Renilla* Luciferase activity from the pRL SV plasmid.

Tube Formation Assay

Tube formation assays were performed essentially as described [29]. Briefly, HUVECs were infected with adenoviruses expressing either human *Gax* (Ad.*hGax*), rat *Gax* (Ad *rGax*), or GFP (Ad.*GFP*) at various multiplicity of infection (MOI) Eighteen hours later 5×10^5 cells were plated on 6 well plates whose surfaces had been coated with reconstituted basement membrane, Low Growth Factor Matrigel, (BD Biosciences, San Jose, CA) and incubated overnight in the presence of serum and 10 ng/ml VEGF$_{165}$ After this, the number of tubes per high powered field were counted for 10 high powered fields, with tubes being defined as a completed connection between cells. Ad GFP-transduced cells were also examined using a fluorescence microscope to demonstrate that GFP was being expressed in the HUVECs forming tubes.

Data Analysis and Statistics

Experiments were repeated 3 or more times For cell culture experiments, at least three wells per experimental group were measured and the mean ± standard deviation determined Statistical significance between the various groups was determined by 2 way ANOVA and the appropriate post-test, with the results being considered statistically significant when $P < 0.05$

RESULTS

Gax is Expressed in Human Vascular Endothelium

Because we hypothesized that Gax is expressed in endothelial cells as well as vascular smooth muscle cells, we first examined *Gax* expression in cultured human vascular endothelial cells and detected *Gax* expression in HUVECs by Northern blot (Fig. 1A) and by RT-PCR using human *Gax*-specific primers (Fig. 1B). Next, to verify that Gax protein is expressed in the endothelium of normal human blood vessels, we subjected a section of human kidney from a nephrectomy specimen to immunohistochemistry with a polyclonal rabbit anti Gax antibody [15] (Fig 2). As expected, *Gax* was expressed in vascular smooth muscle cells. In addition, it was also expressed in the endothelial cells lining the lumen of arteries, as evidenced by nuclear staining of the cells of the intima. From these observations, we conclude that *Gax* is expressed in normal endothelial cells, both *in vitro* and *in vivo*.

Gax Inhibits HUVEC Proliferation *in Vitro*

To test the hypothesis that *Gax* expression inhibits proliferation of endothelial cells, we transduced HUVECs that had been sparsely plated on plastic in 6-well plates with Ad.*hGax* at increasing MOI. Viable cells were counted from each experimental group every 24 h for 4 days. Control cells were transduced with Ad.β-*gal*. Up to MOI = 1000, Ad.β-*gal* did not inhibit HUVEC proliferation (data not shown). Both Ad.*hGax* and Ad.*rGax*, however, inhibited HUVEC and proliferation in a dose-dependent fashion compared to Ad.β-*gal* (Fig 3A and B; $P < 0.05$ for all MOI of virus). Quiescent HUVECs were then transduced with either

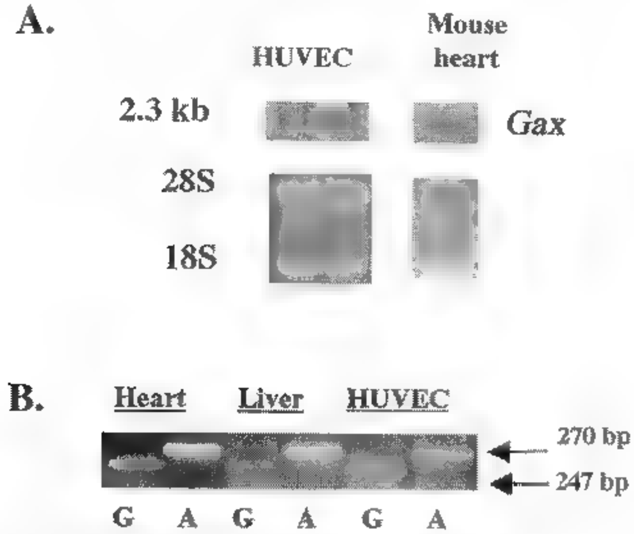

FIG. 1. Gax expression in vascular endothelial cells. Total RNA from HUVECs was subjected to Northern blot with the *Gax* cDNA labeled with ^{32}P by random priming. (A) Northern blots. Two different HUVEC preparations were studied and compared to mouse heart (MH), which is known to express *Gax*. (B) RT PCR. Total RNA from HUVECs was subjected to RT PCR using primers that amplify a 247 bp fragment (base 566 to 812) of the human *Gax* cDNA. The same RT reactions were also subjected to PCR using β-actin primers. See Materials and Methods for details. (G = *Gax*; A = β-actin).

Ad.*hGax* or Ad.*β-gal*, maintained in low serum medium for 24 h, then stimulated with 10% FBS and $VEGF_{165}$ = 10 ng/ml, and 24-h ^{3}H-thymidine uptakes measured (Fig. 4). For comparison, one experimental group was left in low serum medium and is labeled "Quiescent." Consistent with its effect on randomly cycling HUVECs, *Gax* strongly inhibited mitogen-stimulated ^{3}H thymidine uptake ($P < 0.05$ for all MOI of virus). From these results, we conclude that *Gax* expression results in inhibition of HUVEC proliferation, as well as cell cycle arrest.

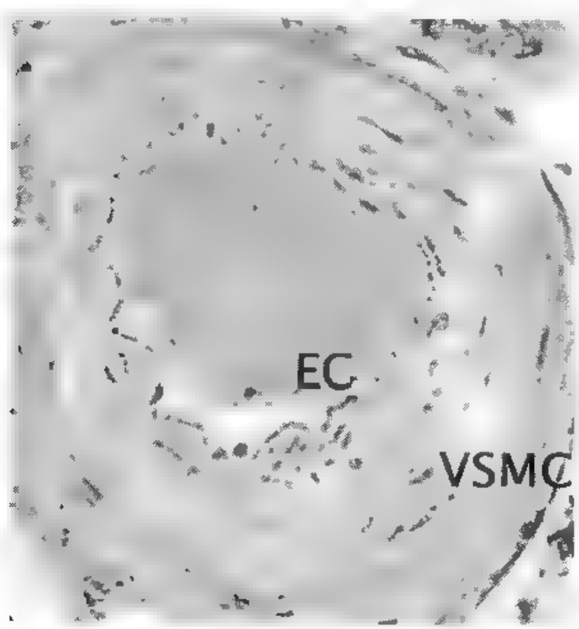

FIG. 2. *Gax* is expressed in both the vascular smooth muscle cells and the endothelial cells of normal human arteries. A section from human kidney obtained from a nephrectomy specimen for renal cell carcinoma was stained with rabbit polyclonal anti-Gax antibody. In the section containing normal kidney, Gax expression was noted in both the media, containing vascular smooth muscle cells (VSMC), as expected from previous studies, but there was also strong staining in the endothelial cells (EC) in the intima lining the lumen.

Gax Activates p21 Promoter Activity in Endothelial Cells

Because *Gax* induces p21 in vascular smooth muscle cells and *Gax* expression inhibited HUVEC proliferation as measured both by cell counts and ^{3}H-thymidine uptake, we tested whether *Gax* could induce p21 expression in endothelial cells. HUVECs were transduced with Ad.*hGax* and Ad.*rGax* at varying MOIs. Cells transduced with an adenovirus expressing green fluorescent protein (Ad.*GFP*) served as controls. By Northern blot, p21 levels were strongly induced in a viral MOI-dependent fashion (Fig. 5A). When cells transduced with Ad.*hGax* in a similar fashion were transfected with a plasmid containing the p21 promoter fused upstream to the firefly Luciferase gene, it was similarly observed that p21 promoter activity was increased by up to 7-fold (Fig. 5B; $P < 0.05$ for all MOI). Transduction with Ad.*GFP* did not affect p21 promoter activity (Fig. 5A and B), nor did transduction with Ad.*β-Gal* (data not shown).

Gax Inhibits Endothelial Cell Tube Formation on Reconstituted Basement Membranes

We next studied the effect of *Gax* expression on angiogenesis *in vitro*. HUVECs were transduced with

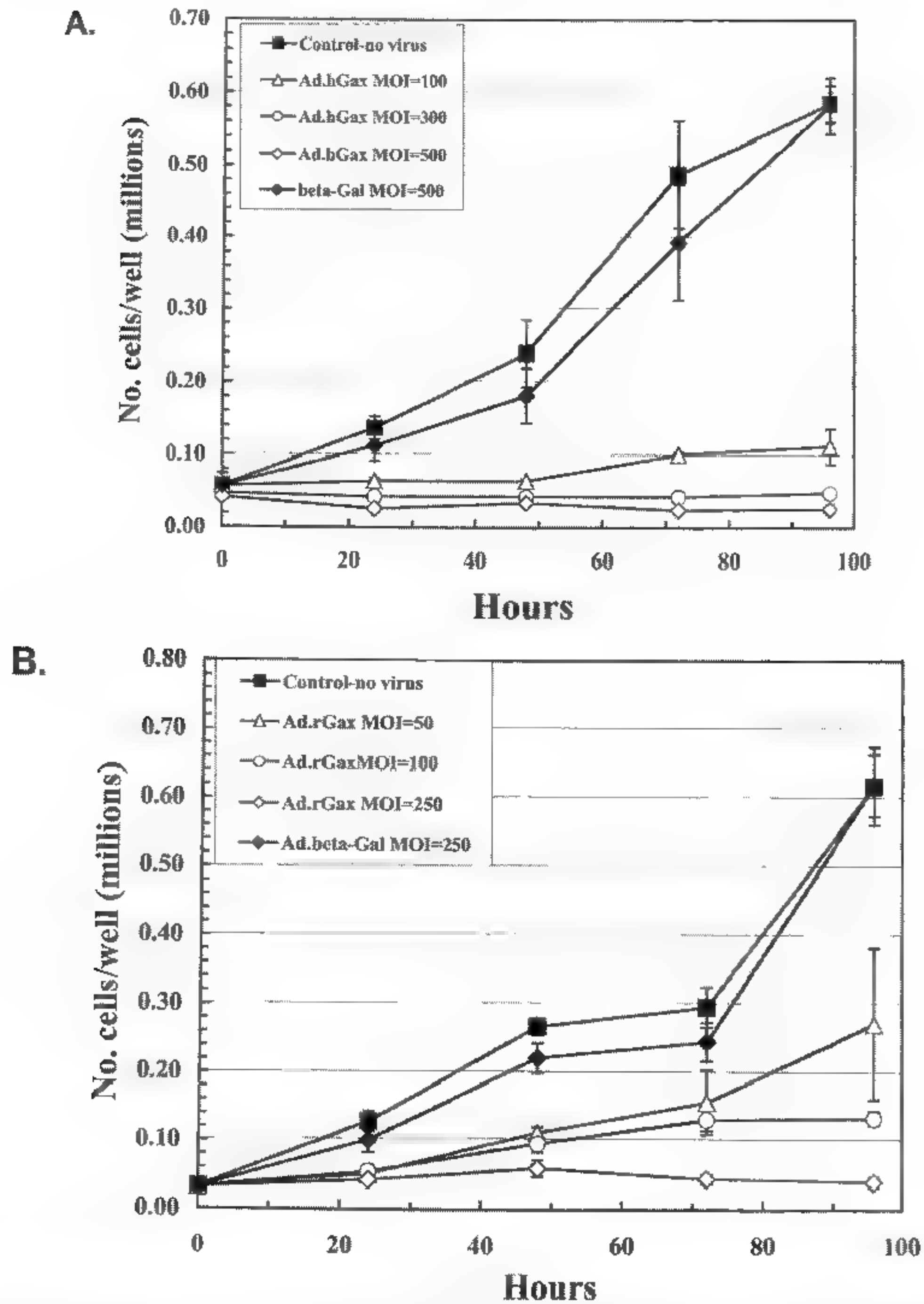

FIG. 3. Inhibition of HUVEC proliferation by *Gax*. Randomly cycling HUVECs growing in 6 well plates in EGM 2 medium were infected with varying MOI of either Ad *hGax*, Ad *rGax*, or Ad *β-Gal*. After infection, 3 wells for each experimental group were trypsinized and counted with cell viability determined by Trypan blue exclusion, and results were counted as mean number of cells + standard deviation. Inhibition of proliferation was statistically significant for all experimental groups at all time points from 48 hours on ($P < 0.05$). (A) Effect of Ad *hGax* on HUVEC proliferation (B) Effect of Ad.*rGax* on HUVEC proliferation

Ad.*hGax* and Ad.*rGax* at varying MOIs and plated on reconstituted basement membrane (Matrigel) in the presence of serum and 10 ng/ml $VEGF_{165}$, conditions that result in robust tube formation. Ad.*GFP* had no effect on tube formation up to MOI = 250, and expression of GFP was verified by fluorescence microscopy (Fig. 6). However, there was a dose-dependent decrease in tube formation beginning at relatively small doses of virus (MOI = 25) and becoming maximal at MOI = 100 (Fig. 6). Maximal inhibition oc-

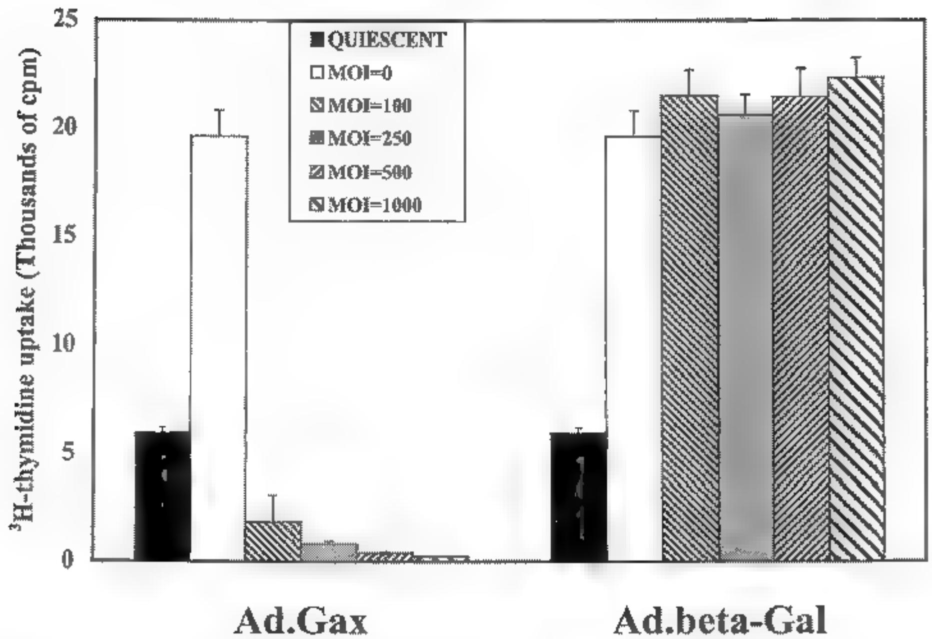

FIG. 4. Inhibition of mitogen induced ^{3}H thymidine uptake in HUVECs by *Gax*. Quiescent HUVECs were transduced with Ad *hGax* at various MOI. Twenty four hours later, the cells were stimulated with serum and $VEGF_{165}$ (10 ng/ml) and 24 h. ^{3}H-thymidine uptakes measured after stimulation. *Gax* strongly inhibited ^{3}H-thymidine uptake in response to mitogen stimulation

curred at a lower MOI than is necessary to maximally inhibit endothelial cell proliferation and activate p21 expression and became maximal at MOI = 50 to 100. We note that is the dose range of virus that we have determined to be necessary to transduce 100% of HUVECs (not shown), implying that few viral particles per cell are necessary to produce sufficient Gax protein to inhibit the cellular machinery that causes tube formation. This is in contrast to the higher viral MOI necessary to produce maximal inhibition of cell cycle progression and induction of p21 expression, implying that more viral particles per cell and therefore a higher level of Gax protein are required to mediate these effects

DISCUSSION

The primary target of proangiogenic factors secreted by tumor cells, and many antiangiogenic factors, is the vascular endothelial cell [1, 30]. During angiogenesis, whether physiologic or tumor induced, endothelial cells undergo distinct changes in phenotype and gene expression, including activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix [1, 4, 31]. Endothelial proliferation accompanies cell invasion and migration, and lumens of new capillaries are formed when endothelial cells adhere to one another and form tubes. Homeobox genes are master regulatory genes with diverse functions in many cell types, both during embryogenesis and in the adult [10–13]. It is therefore not surprising that recently they have been implicated as important transcriptional regulators controlling endothelial cell phenotype during angiogenesis.

Until recently, little was known about how homeobox genes might influence endothelial cell phenotype and behavior during angiogenesis. However, evidence for their involvement in the phenotypic changes endothelial cells undergo during angiogenesis is now accumulating. For instance, Patel *et al.* reported an endothelial cell specific variant of *HOXA9* whose expression is regulated by tumor necrosis factor-α, which is proangiogenic [32]. More direct evidence for the importance of homeobox genes in angiogenesis exists for *HOXD3*. Stimulation of endothelial cells with bFGF induces *HOXD3* expression, as well as integrin $\alpha_v\beta_3$ and the urokinase plasminogen activator, effects that are blocked by *HOXD3* antisense. *In vivo,* sustained expression of *HOXD3* on the chick chorioallantoic membrane retains endothelial cells in an invasive state and prevents vessel maturation, leading to vascular malformations and endotheliomas [33]. In diabetic mice, *HOXD3* expression is impaired in endothelial cells, as is its upregulation after wounding [34] More recently, overexpression of another homeobox gene, HOXB3, in the chick chorioallantoic has been shown to result in an increase in capillary vascular density and angiogenesis, and its blockade by anti sense results in impaired capillary morphogenesis [35].

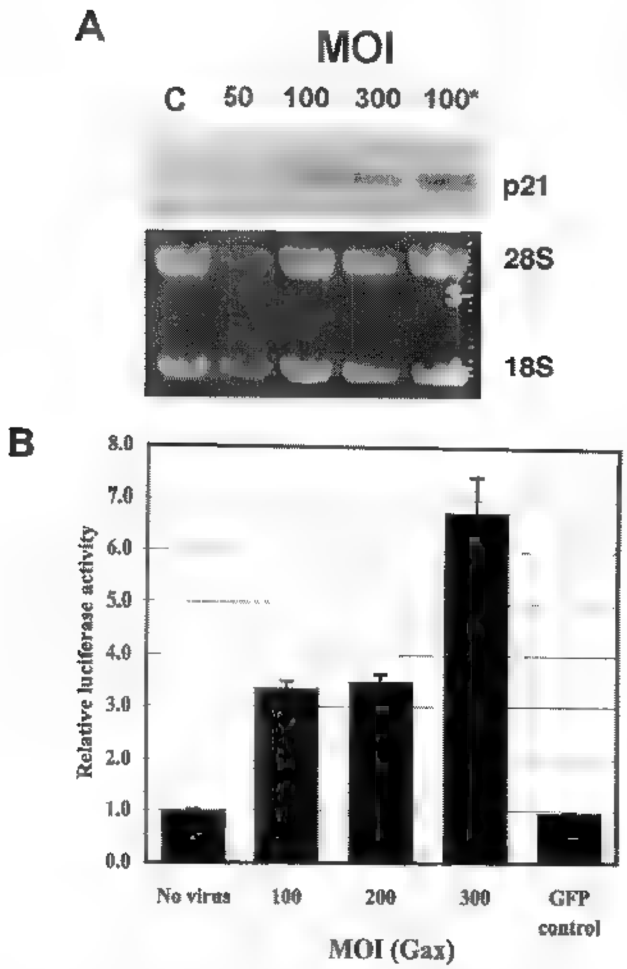

FIG. 5. *Gax* overexpression induces p21 expression (A) *Gax* expression induces p21 expression in HUVECs. Randomly cycling HUVECs were infected with either Ad *hGax* at varying MOIs, Ad.*rGax* at MOI = 100(*), or Ad.*GFP* 300 MOI (C) and then were harvested 24 h later, and Northern blots performed using a p21 probe. (B) Gax expression induces p21 promoter activity HUVECs were infected with Ad.*rGax* and then transfected with a plasmid containing the p21 promoter driving the firefly Luciferase gene. Luciferase activity was measured 24 h later and normalized to *Renilla* Luciferase activity. Error bars represent standard deviation of 3 wells

Taken together, these data suggest significant roles for specific homeobox genes in responding to extracellular signals and activating batteries of downstream genes to induce the phenotypic changes in endothelial cells associated with angiogenesis. These observations are what initially led us to look for additional homeobox genes likely to be involved in the final transcriptional control of genes determining angiogenic phenotype.

In this study, we have reported data strongly suggesting a role for another homeobox gene, the growth arrest homeobox gene *Gax*, in regulating the phenotypic changes that occur in vascular endothelial cells during angiogenesis. Moreover, unlike cell cycle regulators such as p21 or p53, the expression of this gene is relatively restricted to the cardiovascular system [14, 15]. We suspected such a role for *Gax* in endothelial cells during angiogenesis because of its activities in vascular smooth muscle cells, which include G_1 cell cycle arrest [18]; p21 activation [18]; and inhibition of migration towards cytokines and mitogens [19]. We therefore looked for its expression in vascular endothelial cells using RT-PCR, Northern blot, and immunohistochemistry and found that *Gax* is indeed expressed in endothelial cells, both *in vitro* (Fig. 1) and *in vivo* in normal human blood vessels (Fig. 2). Moreover, its expression blocks endothelial cell proliferation, with this inhibition being associated with an upregulation of p21. This upregulation is proportional to the level of expression of *Gax*, and appears to be caused by the activation of the p21 promoter.

Tumor angiogenesis represents a promising new target for anticancer therapy. Given that the most important cell in this process is the vascular endothelial cell, targeting angiogenesis implies targeting vascular endothelial cell processes important to angiogenesis. Specific transcription factors such as *Ets-1* [36] are known to integrate the signals coming from the pathways activated by pro- and antiangiogenic factors and translate these signals to changes in endothelial cell gene expression and phenotype. As such, endothelial cell transcription factors represent both a tool for understanding the phenotypic changes endothelial cells undergo in response to proangiogenic factors secreted by tumor cells that result in angiogenesis and potential targets for the anti-angiogenic therapy of cancer. *Gax* is a homeobox transcription factor originally isolated in vascular smooth muscle cells that has previously been shown to be involved in cardiovascular remodeling [19, 21, 37], inhibiting vascular smooth muscle cell proliferation [18] and migration [19]. We have now shown that *Gax* is also expressed in vascular endothelial cells (Figs. 1 and 2). Moreover, *Gax* inhibits endothelial cell proliferation (Figs. 3 and 4) as well, activating p21 expression (Fig. 5). Of most interest, *Gax* also strongly inhibits tube formation on reconstituted basement membranes (Fig. 6), suggesting that, in addition to its role in inhibiting vascular smooth muscle cell-dependent vascular remodeling processes such as intimal hyperplasia [18, 19], it may also have a role inhibiting vascular remodeling processes that depend mainly on endothelial cells, such as angiogenesis. We therefore conclude that *Gax* may represent an important negative regulator of angiogenesis in vascular endothelial cells, and as such may represent a new molecular tool to understand the transcriptional control of changes in gene expression that occur in endothelial cells during angiogenesis and, more importantly, a potential target for the antiangiogenic therapy of cancer.

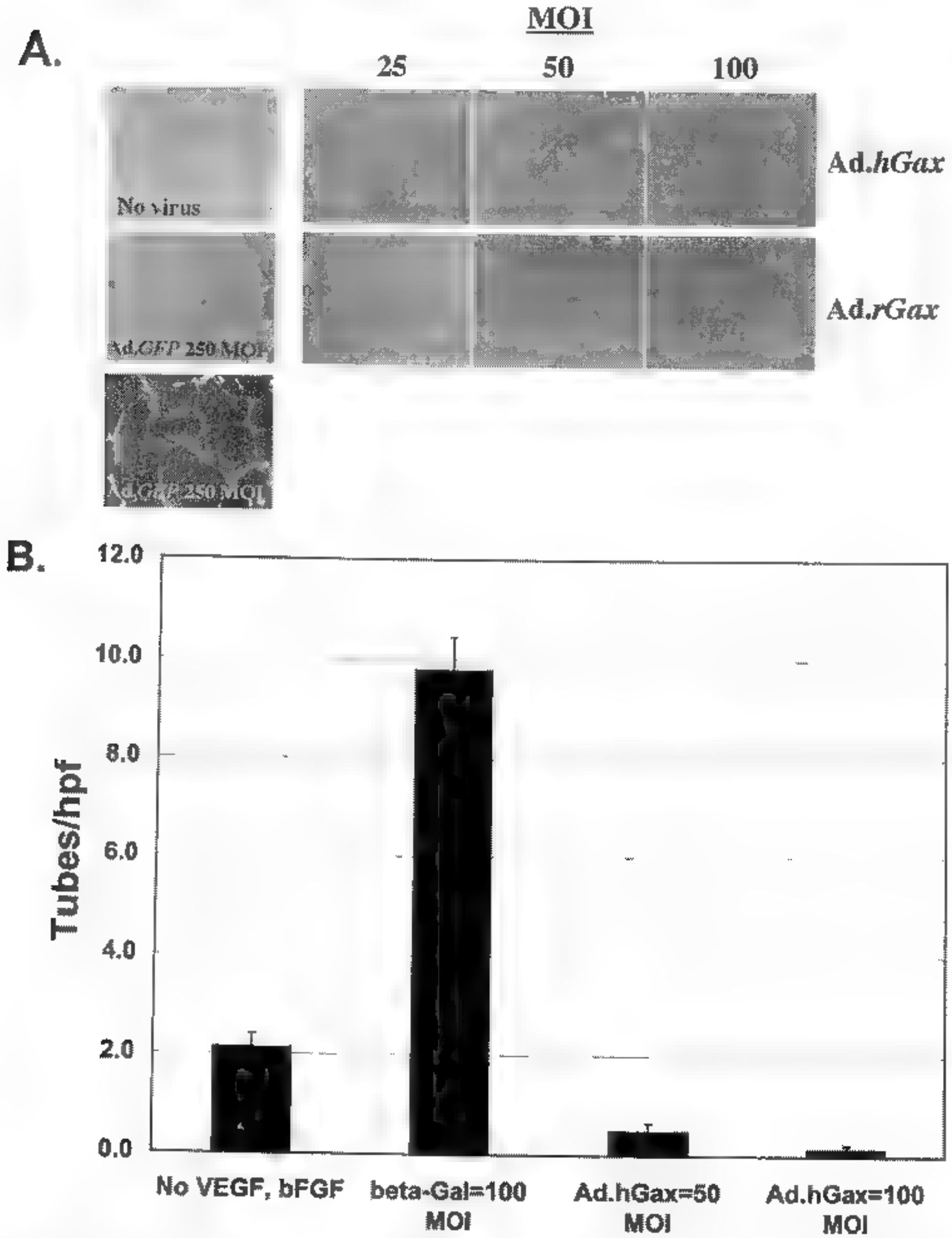

FIG. 6. *Gax* inhibits VEGF induced endothelial cell tube formation on Matrigel. HUVECs were infected with adenoviruses expressing either human *Gax* (Ad.*hGax*), rat *Gax* (Ad.*rGax*) or *GFP* (Ad.*GFP*) at the MOI indicated. Eighteen hours later 5×10^5 cells were plated on Matrigel in 6-well plates and incubated overnight in the presence of serum and 10 ng/ml VEGF. Tube formation was strongly inhibited by both Ad.*hGax* and Ad.*rGax* ($P < 0.05$ at MOI 25). (A) HUVECs in culture demonstrating the inhibition of tube formation by increasing MOI of Ad.*hGax* and Ad.*rGax* and Ad.β-gal was the control. (B) Tube counts for an experiment in which Ad.*hGax* was used to inhibit endothelial cell tube formation.

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ELSEVIER

BRIEF REVIEWS

Control of Vascular Cell Differentiation by Homeobox Transcription Factors

David H. Gorski* and Kenneth Walsh

Homeobox genes are a family of transcription factors with a highly conserved DNA-binding domain that regulate cell proliferation, differentiation, and migration in many cell types in diverse organisms. These properties are responsible for their critical roles in regulating pattern formation and organogenesis during embryogenesis. The cardiovascular system undergoes extensive remodeling during embryogenesis, and cardiovascular remodeling in the adult is associated with normal physiologic processes such as wound healing and the menstrual cycle, and disease states such as atherosclerosis, tumor-induced angiogenesis, and lymphedema. Aside from their roles in the formation of the embryonic vascular system, homeobox genes recently have been implicated in both physiologic and pathologic processes involving vascular remodeling in the adult, such as arterial restenosis after balloon angioplasty, physiologic and tumor-induced angiogenesis, and lymphangiogenesis. Understanding how homeobox genes regulate the phenotype of smooth muscle and endothelium in the vasculature will improve insight into the molecular mechanisms behind vascular cell differentiation and may suggest therapeutic interventions in human disease.
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Changes in cellular phenotype leading to remodeling in the vascular system occur during normal development and in pathologic states. During embryogenesis, vascular endothelial cell (EC) precursors converge into blood islands, which ultimately develop into the aortic arches and capillary networks that provide oxygen and nutrients to the developing organs and limbs. From this, lymphatic EC precursors bud from embryonic veins to form the lymphatic vascular system. In the adult, examples of changes in vascular cell phenotype leading to vascular remodeling include wound healing and the menstrual cycle, during which both angiogenesis and regression of blood vessels are tightly regulated. Examples of pathologic remodeling include atherosclerosis and arterial restenosis after balloon angioplasty. In both processes, vascular smooth muscle cells (VSMCs) migrate from the media to the intima and proliferate, leading to narrowing of the arterial lumen and the subsequent complications, including hypoxia or even anoxia in downstream tissues (Ross 1993)—quickly in the case of restenosis and slowly in the case of atherosclerosis. In addition, phenotypic changes in vascular ECs leading to vascular remodeling play a critical role in tumor biology because diffusion of oxygen and nutrients limits tumor growth to within 1 mm of a capillary. To overcome this limitation, tumors secrete proangiogenic factors to stimulate the ingrowth of new blood vessels (Folkman 1995), which develop from ECs with an immature phenotype (Eberhard et al. 2000). Similarly, tumors also secrete prolymphangiogenic factors, which allow for the ingrowth of lymphatics and subsequent metastasis to regional lymph nodes (Skobe et al. 2001). Thus, understanding the mechanisms underlying the phenotypic changes that lead to vascular remodeling could produce insights into diseases as diverse as atherosclerosis or restenosis, lymphedema, and cancer.

Although the receptors and signaling pathways activated by growth factors and cytokines have been studied extensively in the vascular system, much less is known about the molecular biology of the downstream transcription factors activated by these pathways to regulate tissue-specific gene expression controlling the growth and differentiation of these cells. Transcription factors represent a common mechanism that can integrate multiple signaling pathways to produce the necessary changes in gene expression and phenotype for vascular cells to perform their functions. Homeobox genes encode a family of transcription factors

containing a common 60-amino-acid DNA-binding motif known as the homeodomain, containing a helix–turn helix motif similar to that found in prokaryotic regulatory proteins such as Cro, CAP, and the λ repressor in *Escherichia coli* (Scott et al. 1989). They are regulators of cell differentiation, proliferation, and migration in both vertebrates and invertebrates, controlling pattern formation in the embryo and organogenesis, as well as oncogenesis in the adult (Cillo et al 1999, Ford 1998, Krumlauf 1994). Given these characteristics, homeobox genes are excellent candidates for important roles in the final transcriptional regulation of genes responsible for vascular remodeling and angiogenesis in normal physiology and disease. Recently several homeobox genes have been implicated in the phenotypic changes in vascular cells that lead to intimal hyperplasia, arterial restenosis after angioplasty, angiogenesis, and lymphangiogenesis. It is therefore an opportune time to review briefly what is currently known about homeobox gene expression and activity during vasculogenesis and vascular remodeling in the adult

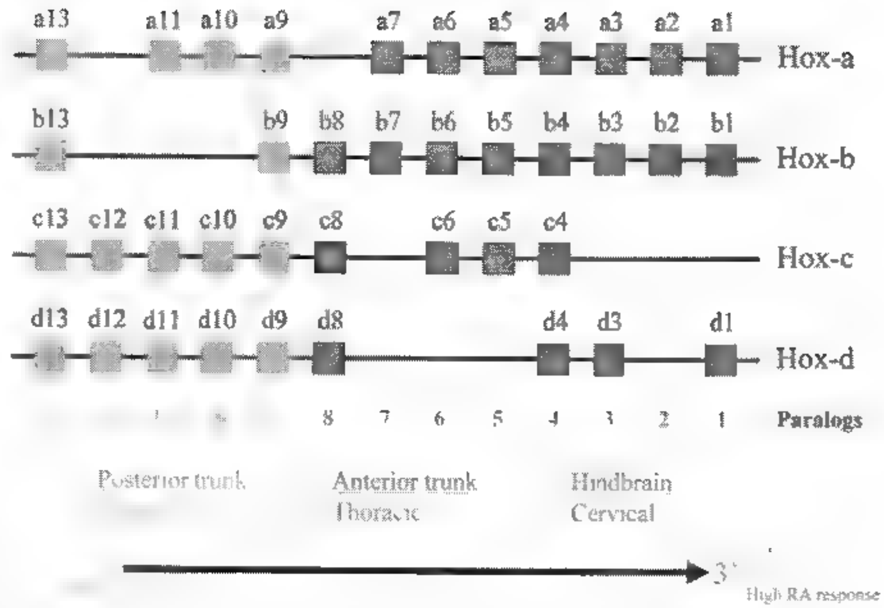

Figure 1. Organization of the HOX clusters. The four HOX clusters in the human and mouse are believed to have evolved through gene duplication. In the human, there are 39 homeobox genes in the HOX clusters (Kosaki et al. 2002). In the mouse, as shown in this figure, the 3′ genes are expressed early in embryogenesis in the more rostral regions of the embryo, whereas the 5′ genes are expressed later in embryogenesis in the caudal regions of the embryo (Cillo et al. 1999). The 3′ rostral genes are highly responsive to retinoic acid (RA), whereas the 5′ caudal genes are less sensitive. Each homeobox gene can have as many as three paralogs in the same position in other HOX clusters Each HOX cluster is located on a different chromosome. The arrangement of the human HOX clusters, HOX A through D, is nearly identical to the mouse See text for details.

• Homeobox Gene Expression and Function During Vascular Development

HOX Cluster Genes

In *Drosophilia melanogaster* and vertebrates, many, but not all, homeobox genes are arranged in gene clusters. In mice and humans, there are four unlinked complexes—HOX A through HOX D—that arose from gene duplication (Krumlauf 1994). Because of this, each HOX gene may have as many as three paralogues. The location of each HOX gene in the cluster corresponds to its axial pattern of expression in the developing embryo, with 5′ genes expressed more toward the caudal region and 3′ genes expressed more toward the rostral region (Figure 1), with specific embryonic defects due to knockouts of specific HOX genes occurring in the axial region of their expression. HOX genes have been studied widely with regard to their ability to control pattern formation in the developing embryo. They are powerful regulators of pattern formation, as evidenced by the homeotic mutations (i.e., mutations in which one normal body part is substituted for another normal body part, as in *Antennapedia*)

Several members of the HOX clusters are expressed in the cardiovascular system during embryogenesis, including *HOXA5*, *HOXA11*, *HOXB1*, *HOXB7*, and *HOXC9* (Miano et al. 1996). Moreover, there is functional evidence for involvement of HOX genes in vasculogenesis. For example, transgenic mice with null mutations of the *HOXA3* gene die shortly after birth, suffering from defects in the cardiovascular system that include heart-wall malformations, persistent patent ductus arteriosus, and aortic stenosis (Chisaka and Capecchi 1991). In some of these mice, the right carotid artery fails to form, and in all mice the aorta is thin walled and poorly developed. The overall constellation of defects in *HOXA3* null mice is similar to that observed in the human congenital disorder DiGeorge syndrome (Chisaka and Capecchi 1991).

Because paralogous HOX genes have similar DNA binding domains and axial expression patterns during embryogenesis, it has been hypothesized that they may have overlapping or complementary functions. Thus, targeting one paralogue may not produce an observable phenotype. This has been demonstrated by antisense targeting of the messages for the paralogous HOX 3 group (*HOXA3* and *HOXB3*), which results in the regression of aortic arch 3 in a manner similar to that of arch 2 (Kirby et al. 1997). Similarly, targeting paralogous group 5 genes (*HOXA5*, *HOXB5*, and *HOXC5*) causes the appearance of an additional pharyngeal arch containing a novel and aortic arch artery (Kirby et al. 1997). These observations suggest that paralogues probably have overlapping functions in vascular development and that in at least some cases they can compensate for each other when the function of one is impaired.

Paired Related Genes

The expression of two genes not located in the HOX clusters—*Prx1* (formerly known as *MHox* or *Phox*) (Cserjesi et al. 1992) and *Prx2* (formerly known as *S8*) (Opstelten et al. 1991)—during embryogenesis suggests that they have an important role in vasculogenesis. In the vascular

system, expression of *Prx1* and *Prx2* is associated with the primary vessel wall and becomes increasingly restricted to the adventitial and outer medial cell layers as development proceeds (Bergwerff et al. 1998). *Prx1* expression colocalizes with procollagen I and fibrillin 2 but not with smooth muscle α actin, whereas *Prx2* expression is highly associated with the developing ductus arteriosus and is one of the earliest markers of its differentiation. Transgenic mice with null mutations *Prx1* and *Prx2* suggest their relative importance in vascular patterning in the embryo. *Prx2*$^{-/-}$ mutants do not show cardiovascular malformations. In contrast, *Prx1*$^{-/-}$ mutants display abnormal positioning and awkward curvature of the aortic arch, in addition to a misdirected and elongated ductus arteriosus (Bergwerff et al. 2000). However, *Prx1*$^{-/-}$/*Prx2*$^{-/-}$ double mutants demonstrate a more severe form of these abnormalities, some of them possessing an anomalous retroesophageal right subclavian artery, as well as excessive tortuosity of all great vessels as they run through the mesenchyme, although they do not have cardiac anomalies (Chesterman et al. 2001). Thus, the loss of *Prx2* function exacerbates anomalies due to the loss of *Prx1*, suggesting functional overlap between these two genes in vascular development.

Hex: *An Early Marker of EC Precursors and Regulator of EC and VSMC Differentiation*

Hex is a proline-rich divergent homeobox gene originally isolated from hematopoietic tissues (Crompton et al. 1992). Expressed in a range of hematopoietic progenitor cells and cell lines (Crompton et al. 1992), *Hex* is an early marker of EC precursors and is transiently expressed in the nascent blood islands of the visceral yolk sac and later in embryonic angioblasts and endocardium (Thomas et al. 1998). The *Xenopus laevis* homologue *XHex* is expressed in vascular ECs throughout the developing vascular network, and its overexpression leads to disruption of vascular structures and an overall increase in EC number (Newman et al. 1997). These observations suggest an important role for *Hex* in the vascular patterning due to the migration and proliferation of EC precursors. In addition, it has been reported recently that *Hex* also is expressed in VSMCs (Sekiguchi et al. 2001). Its expression is upregulated in neointimal VSMCs after balloon injury in the rat, and *Hex* activates the promoter of NMHC-B/SMemb, a nonmuscle-specific isoform of the smooth muscle myosin heavy chain that is expressed during embryonic development of the aorta, declines in the neonate and adult, and is re-induced in vascular lesions.

Given the above experimental observations, it has been assumed that *Hex* promotes the conversion of ECs to the angiogenic phenotype. However, recent evidence does not support that assumption and suggests that the role of *Hex* in controlling vascular phenotype may be more complex than first thought. First, disruption of the *Hex* gene in mouse embryos does not produce a detectable change in the vascular phenotype (Barbera et al. 2000), suggesting that other factors—perhaps the transcription factor *Scl* (Liao et al. 2000)—may compensate for the loss of *Hex* function. Also, it has been reported recently that *Hex* overexpression in human umbilical vein ECs (HUVECs) inhibits in vitro surrogates for angiogenesis, including migration toward vascular endothelial growth factor (VEGF), invasion, proliferation, and tube formation on reconstituted basement membrane (Matrigel) (Nakagawa et al. 2003). In addition, *Hex* was shown to inhibit the expression of angiogenesis-related membrane genes, including those encoding VEGFR-1, VEGFR-2, neuropilin 1, integrin subunit α_v, Tie-1, and Tie-2. It remains to be clarified whether *Hex* inhibits angiogenesis in vivo, but, taken together with previous reports, these observations suggest a complex role for *Hex* in regulating the proliferation and development of the vascular tree and the differentiation of ECs and VSMCs.

Prox1 *and Development of the Lymphatic System*

The lymphatic system is a vascular network of thin-walled capillaries and larger vessels lined by a layer of ECs that drain lymph from the tissue spaces of most organs and return it to the venous system for recirculation. Early in development, primitive lymph sacs develop from endothelial budding from the veins to form the lymphatic system. The homeobox gene *Prox1* has been implicated in the development of the lymphatic system. Originally isolated by its homology to the *Drosophila* gene *prospero* (Oliver et al. 1993), *Prox1* has an expression pattern that suggests a functional role in a variety of tissues, including eye lens, central nervous system, and liver, with null mutations leading to embryonic lethality (Wigle and Oliver 1999). Supporting a role in lymphatic development is the observation that *Prox1* is the earliest marker of lymphatic EC precursors, and in *Prox1*$^{-/-}$ knockout mice, budding of ECs that give rise to the lymphatic system is arrested at embryonic day 11.5, resulting in mice without lymphatic vasculature (Wigle and Oliver 1999). In contrast, vasculogenesis and angiogenesis are unaffected by the loss of *Prox1* function (Wigle and Oliver 1999, Wigle et al. 2002). In addition, expression of *Prox1* in vascular ECs results in proliferation and a reprogramming of these cells to a lymphatic EC phenotype, inducing expression of lymphatic genes such as *VEGFR-3*, *p57*^{kip2}, and *desmoplakin I/II* and downregulating vascular EC genes such as *STAT6* and *neuropilin 1* (Hong et al. 2002, Petrova et al. 2002). Moreover, this lymphatic reprogramming due to *Prox1* expression occurs only in vascular ECs, although *Prox1* is still able to induce cyclin expression and proliferation in other cell types (Petrova et al. 2002). Together, these data suggest a role for *Prox1* as a general inducer of proliferation and a key regulatory gene in the developing lymphatic system.

• Homeobox Gene Expression and Function in Mature Blood Vessels

Homeobox Gene Expression during VSMC Phenotypic Modulation and Vascular Disease

VSMCs exist within a spectrum of phenotypes ranging from the "contractile" to the "synthetic" state (Ross 1993). Cells in the contractile state are quiescent; do not migrate, are relatively insensitive to mitogens; express contractile proteins, including smooth muscle-specific isoforms of actin and myosin; and are associated with normal vessel wall. Synthetic state cells, on the other hand, are able to migrate; express lower levels of contractile proteins, with higher levels of nonmuscle isoforms of myosin and actin; secrete extracellular matrix components, and generally resemble less-differentiated VSMCs found in fetal blood vessels. Over the last decade, evidence has been accu-

mulating that homeobox genes are involved in regulating the transition between these two phenotypes.

In the adult, several members of the HOX clusters are expressed in the cardiovascular system. Homeobox sequences isolated from adult rat aorta include *HOXA2*, *HOXA4*, *HOXA5*, and *HOXB7*, and *HOXA11* (Gorski et al. 1994, Patel et al. 1992). Other groups have reported the expression of *HOXA5*, *HOXA11*, *HOXB1*, *HOXB7*, and *HOXC9* in human adult and fetal aortic smooth muscle (Miano et al. 1996, Patel et al. 1992). Of these, *HOXB7* and *HOXC9* are expressed at markedly higher levels in embryonic VSMCs compared with adult VCMCs, suggesting a role in the proliferation and remodeling that occur during embryogenesis (Miano et al. 1996). In addition, overexpression of *HOXB7* in C3H10T1/2 cells results in increased proliferation; the induction of a VSMC-like morphology; and the expression of early, but not intermediate, VSMC markers. Moreover, *HOXB7* mRNA was detected in human atherosclerotic plaques at a higher level than in normal human arterial media (Bostrom et al. 2000). These observations suggest a role for *HOXB7* and perhaps *HOXC9* in vascular remodeling, either in the expansion of immature VSMCs or the change of vascular myocytes to a more immature phenotype, both of which occur in human vascular diseases, such as atherosclerosis and restenosis after balloon angioplasty.

Gax *and Control of Smooth Muscle Phenotype*

Originally isolated from a rat aorta cDNA library with the use of degenerate oligonucleotide probes directed at the most conserved protein sequence of the *Antennapedia* homeodomain (Gorski et al. 1993a), *Gax* (also known as *Mox-2*) encodes a homeodomain-containing transcription factor whose expression has multiple effects on vascular phenotype. Although its expression is more widespread in the embryo, including all three muscle lineages and brain (Skopicki et al. 1997), *Gax* expression in the adult is more narrowly confined to cardiovascular tissues, including heart, medial smooth muscle cells of arteries, lung, and mesangial cells in the kidney (Gorski et al. 1993a). In VSMCs, *Gax* expression is downregulated rapidly by mitogenic signals such as serum, platelet-derived growth factor (Gorski et al. 1993a), and angiotensin II (Yamashita et al. 1997), and more slowly upregulated by growth arrest signals such as serum deprivation (Gorski et al. 1993a) and C-type natriuretic peptide (Yamashita et al. 1997). Moreover, *Gax* expression is also downregulated in the proliferating VSMCs of the rat carotid artery after balloon injury (Weir et al. 1995). *Gax* expression induces G_0/G_1 cell-cycle arrest and upregulates p21 expression by a p53-independent mechanism, and it is this upregulation of p21 that accounts for its antiproliferative activity (Smith et al 1997). *Gax* also controls the migration of VSMCs toward chemotactic growth factors through its ability to alter integrin expression, downregulating integrins $\alpha_V\beta_3$ and $\alpha_V\beta_5$ through the specific suppression of the β_3 and β_5 subunits, both in vitro and in vivo (Witzenbichler et al. 1999). Cell-cycle arrest, which does not by itself suppress VSMC migration, is essential for the antimigratory activity of *Gax*, as *Gax* overexpression has no effect on p21$^{-/-}$ cells Collectively, these data suggest that *Gax* may function to coordinate vascular cell growth and motility through its ability to regulate integrin expression in a cell cycle-dependent manner. The ability of *Gax* to induce apoptosis in proliferating VSMCs (Perlman et al. 1998) is consistent with these observations, because integrin signaling is an important regulator of cell viability.

Control of Smooth Muscle Phenotype by Prx

The expression of *Prx1* and *Prx2* cannot be detected in the vasculature of adult rats, but they are upregulated in rat pulmonary arteries in which pulmonary hypertension was induced by the injection of monocrotaline (Jones et al. 2001). Induction of *Prx1* and *Prx2* expression in vitro and in vivo is coincident with induction of the extracellular matrix protein tenascin C, which promotes growth and survival of cultured VSMCs. *Prx1* activates the tenascin-C promoter and induces VSMC proliferation in vitro. Consistent with these observations, *Prx1* is upregulated by angiostatin II and, along with the serum response factor, mediates angiotensin II-induced smooth muscle α-actin expression in VSMCs (Hautmann et al 1997). Collectively, it appears that *Prx1* and *Prx2* genes have roles both in regulating the proliferation of embryonic VSMCs during the formation of the vascular system and in controlling the change of mature VSMCs to a more immature phenotype that occurs in some vascular diseases.

Homeobox Genes and Postnatal Angiogenesis

Functional evidence for the involvement of HOX cluster genes in the regulation of the angiogenic phenotype comes from the study of the paralogous HOX genes *HOXD3* and *HOXB3*, each of which appears to have distinct and complementary roles in this process *HOXD3* is expressed at high levels in proliferating ECs induced to form tubes on Matrigel but not in quiescent ECs, and its expression is induced by basic fibroblast growth factor (bFGF) (Boudreau et al. 1997) Functionally, blocking *HOXD3* expression with antisense inhibits the bFGF-stimulated upregulation of integrin $\alpha_V\beta_3$ and urokinase plasminogen activator (uPA) without affecting EC proliferation. In contrast, overexpressing *HOXD3* leads to expression of these genes and a morphologic change to the angiogenic phenotype, resulting in the formation of endotheliomas in vivo. In diabetic mice, *HOXD3* expression is impaired in ECs, as is its upregulation after wounding, suggesting that impaired *HOXD3* expression might be involved in the impaired wound healing observed in diabetics (Uyeno et al. 2001). In addition, the *HOXD3* paralogue, *HOXB3*, has been reported to influence angiogenic behavior in a manner distinct from *HOXD3*. Antisense against *HOXB3* impairs the capillary morphogenesis of dermal microvascular ECs and decreases the phosphorylation of the Eph A2 receptor (Myers et al. 2000). Consistent with this result, constitutive expression of *HOXB3* results in an increase in capillary vascular density and angiogenesis, but does not produce endotheliomas. Taken together, these results suggest overlapping and complementary roles for *HOXB3* and *HOXD3* in angiogenesis, with *HOXD3* promoting the invasive or migratory behavior of ECs in response to angiogenic signals and *HOXB3* promoting capillary morphogenesis of these new vascular sprouts.

In contrast to *HOXB3* and *HOXD3*, another HOX cluster gene—*HOXD10*—

inhibits EC conversion to the angiogenic phenotype. Expression of *HOXD10* is higher in quiescent endothelium as compared with tumor-associated vascular endothelium. Moreover, sustained expression of *HOXD10* inhibits EC migration and blocks bFGF- and VEGF-induced angiogenesis in the chick chorioallantoic membrane assay in vivo. Consistent with these observations, human ECs overexpressing *HOXD10* fail to form new blood vessels (Myers et al. 2002) when embedded in Matrigel-containing sponges (Nor et al. 2001) in nude mice. In addition, human ECs overexpressing *HOXD10* express a gene profile consistent with a quiescent, nonangiogenic state, with decreased expression of genes that influence remodeling of the extracellular matrix and cell migration during angiogenesis, such as the uPA receptor and the α_3 and β_4 integrin subunits (Myers et al. 2002). Based on these observations, coupled with the proangiogenic activity of *HOXB3* and *HOXD3*, it has been proposed that the 5′ and 3′ HOX genes have distinct influences on EC behavior, with the more 3′ genes tending to promote the angiogenic phenotype and the more 5′ HOX genes such as *HOXD10* tending to be inhibitory to the angiogenic phenotype and dominant.

The expression of other members of the HOX clusters also have been detected in vascular ECs. One example is *HOXA9EC*, an alternatively spliced variant of HOXA9 whose expression is downregulated by tumor necrosis factor α (TNF-α), which, in addition to its numerous other activities, is proangiogenic (Patel et al. 1999). Also, the expression of several members of the HOX B cluster in HUVECs is regulated by VEGF and tissue plasminogen activator, but not bFGF (Belotti et al. 1998). Because HOX B cluster gene expression does not correlate with the mitogenic state of the cell but rather is altered with the state of cellular differentiation, it has been suggested that these genes are involved in the morphogenic changes associated with the angiogenic phenotype.

Recently it has been reported that *Gax* also is expressed in vascular ECs (Gorski and Leal 2003). As in VSMCs, in ECs, *Gax* expression results in cell-cycle arrest and induces p21 expression and promoter activity. Of note it also strongly inhibits EC tube formation in response to VEGF on Matrigel (Gorski and Leal 2003) in a manner similar to that of *Hex* (Nakagawa et al. 2003). These additional observations suggest that in addition to its likely role in maintaining VSMCs in the contractile phenotype, *Gax* may also have a role in EC differentiation. Taken together, all of the above observations suggest that *Gax* may be a global inhibitor of vascular cell activation. However, like *Hex* knockout mice (Barbera et al. 2000), mice transgenic for a null mutation in *Gax* have not been reported to show vascular anomalies (Mankoo et al. 1999). Rather, they show skeletal muscle anomalies in the limbs and die shortly after birth from unknown causes. This would tend to suggest that other homeobox factors, such as *Mox 1* (Candia and Wright 1996) or possibly *Pax3* (Stamataki et al. 2001), might compensate for a lack of *Gax/Mox-2* expression in the developing cardiovascular system. It would be of great interest to determine whether *Gax* knockout mice demonstrate increased angiogenesis in response to proangiogenic stimuli, but such studies would be difficult because of their very brief life span. Similar studies would also be of interest in *Hex* knockout mice.

Other homeobox genes also are likely to be involved in regulating angiogenesis, whether physiologic or tumor induced. For example, St. Croix et al. (2000) used serial analysis of gene expression to look for expressed sequence tags (ESTs) whose expression is at least 10-fold greater in tumor endothelium compared with normal endothelium. Not surprisingly, many of the ESTs they reported derive from extracellular matrix proteins. However, one EST was similar to the homeobox gene *Dlx-3*, a member of the *Distal less* family of homeobox genes. This EST was not detectable in the developing corpus luteum, implying a distinction between tumor angiogenesis and physiologic angiogenesis. Interestingly, *Dlx-3* has been implicated in placental function (Beanan and Sargent 2000). Other placental homeobox genes include *Dlx-4*, *Gax/Mox-2*, *HB24*, and *Msx2* (Quinn et al. 1997). Given the critical importance of angiogenesis and blood vessel regression in placental function, it is reasonable to predict that some of these genes are involved in vascular remodeling in the placenta. It is also reasonable to postulate that homeobox genes previously demonstrated to be important in inducing proliferation and migration of ECs and EC precursors during angiogenesis—such as *Hex*—also may be important in inducing angiogenesis in the adult vasculature.

▪ Conclusions

Although much more is known since the last time we reviewed the expression and function of homeobox genes in the vasculature (Gorski et al. 1993b), knowledge of the transcriptional regulation of VSMC and EC phenotype still is not as detailed as is the understanding of the cytokines and growth factors that act on ECs and VSMCs to regulate their phenotype, the receptors these factors activate, and the downstream signaling pathways activated in turn by these receptors. However, a growing number of homeobox genes have been implicated in vascular development in the embryo and vascular remodeling, angiogenesis, and vascular diseases in the adult. Moreover, with the description of *Prox1* (Hong et al. 2002, Petrova et al. 2002), it has become clear that homeobox genes participate in the development of the lymphatic vascular system as well. Given the sheer number of homeobox genes and potential interactions between them and vascular remodeling, it is difficult to generalize too much about the roles of homeobox genes in these processes, some of which are listed in Table 1. It is possible, however, to come to three general conclusions with regard to how homeobox genes regulate vascular remodeling.

1. Pathways controlled by homeobox genes are redundant, especially during embryogenesis. This implies that it is more likely to be the overall pattern of homeobox gene expression rather than any one individual homeobox gene that regulates the phenotype of VSMCs and ECs during angiogenesis and vascular remodeling. The roles of *HOXB3*, *HOXD3*, and *HOXD10* in regulating EC phenotype during angiogenesis represent a good example of this principle. It may be the balance between pro- and antiangiogenic HOX cluster genes that determine whether an EC becomes angiogenic, and different proangiogenic HOX genes may control different stages or aspects of angiogenesis (e.g., *HOXB3* and *HOXD3*). It also can be postulated that *Gax* and *Hex* help to determine this balance. Similarly, in VSMCs, it can be postulated that the balance between *Gax* and *Prx1/Prx2* (and possibly *Hex*) plays a major role in

Table 1. Homeobox genes expressed in the cardiovascular system

Cell type	*Gene*	*Function/observation*	*Reference*
VSMC	*Gax (Mox-2)*	Downregulated upon mitogen stimulation and vascular injury Causes G_1 cell-cycle arrest and inhibits VSMC migration Inhibits integrin $\alpha_V\beta_3$ and $\alpha_V\beta_5$ expression Induces apoptosis in cycling cells Inhibits restenosis after balloon injury Interacts with *Pax3*	Gorski et al. 1993a, Perlman et al. 1998, Smith et al. 1997, Stamataki et al. 2001, Weir et al. 1995, Witzenbichler et al. 1999, Yamashita et al. 1997
	Hex	Induces expression of immature actin isoform in VSMCs	Sekiguchi et al. 2001
	HOX B7	More highly expressed in fetal VSMCs than in adult VSMCs Induces differentiation of C3H10T1/2 cells into VSMC-like cells	Bostrom et al. 2000, Miano et al. 1996
	HOX C9	More highly expressed in fetal VSMCs than in adult VSMCs	Miano et al. 1996
	HOX A3 and *B3*	*HOX A3* knockout mice have vascular anomalies Blocking *HOX A3* and *B3* causes regression of aortic arch 3	Kirby et al. 1997
	HOX A5, B5, and *C5*	Blocking expression causes appearance of additional aortic arch artery	Kirby et al. 1997
	HOX A2, A4, A11, and *B1*	Isolated from vascular smooth muscle, functions in VSMC unknown	Gorski et al. 1993a and 1994, Patel et al. 1992
	Prx1	Interacts with serum response factor to activate binding Putative role in angiotensin II-mediated smooth-muscle α-actin expression *Prx1/Prx2* double-null mutants demonstrate vascular anomalies Activates proliferation and tenascin-C expression	Bergwerff et al. 1998 and 2000, Chesterman et al. 2001, Hautmann et al. 1997, Jones et al. 2001
	Prx2	Widely expressed in embryonic vasculature *Prx1/Prx2* double-null mutants demonstrate vascular anomalies	Bergwerff et al. 1998 and 2000, ten Berge et al. 1998
Vascular ECs	*HOXA9EC*	EC specific, function presently unknown Expression modulated by tumor necrosis factor α	Patel et al. 1999
	HOX B cluster	HOX B cluster induced by differentiating factors	Belotti et al. 1998
	HOXB3	Involved in regulating capillary morphogenesis	Myers et al. 2000
	HOXD3	Induces expression of integrin $\alpha_V\beta_3$ Induces angiogenic phenotype in ECs Impaired function associated with impaired wound healing	Boudreau et al. 1997, Uyeno et al. 2001
	HOXD10	Inhibits angiogenesis and changes EC gene expression profile to the nonangiogenic state	Myers et al. 2002
	Dlx-3	Expressed sequence tags with homology to *Dlx-3* expressed at high levels in tumor endothelium Necessary for placental development	Quinn et al. 1997, St Croix et al. 2000
	Gax (*Mox-2*)	Inhibits in vitro surrogates for angiogenesis May have function in placental–mesenchymal interactions	Gorski and Leal 2003, Quinn et al. 1997 and 2000
	Hex	Early marker of ECs during embryogenesis Expressed throughout the vascular network Overexpression increases EC number in embryos Overexpression blocks EC tube formation on Matrigel	Barbera et al. 2000, Liao et al. 2000, Nakagawa et al. 2003, Newman et al. 1997, Sekiguchi et al. 2001, Thomas et al. 1998
Lymphatic ECs	*Prox1*	Specific to lymphatic ECs Induces expression of lymphatic EC-specific genes Null mutations prevent development of lymphatic system Master regulator of lymphatic vessel formation from embryonic venous system	Hong et al. 2002, Petrova et al. 2002, Wigle and Oliver 1999, Wigle et al. 1999 and 2002

EC, endothelial cell. VSMC, vascular smooth muscle cell.

determining whether VSMCs become contractile or synthetic.

2. Individual homeobox genes may function as master regulatory genes for parts of the vascular system. For instance, although a master regulatory gene controlling development of angioblasts into vascular ECs or VSMCs remains to be identified, *Prox1* represents a very good candidate for such a role in lymphatic endothelium. However, it must be remembered that most homeobox genes controlling vascular phenotype also are expressed in other tissues. Even *Prox1* is expressed in liver and eye lens during embryogenesis. Similarly, *Prx1* is clearly important in skeletal development (ten Berge et al. 1998), and *Gax* is important in skeletal muscle development (Mankoo et al. 1999). This implies that cell-type-specific factors influence the activities of homeobox genes in both ECs and VSMCs and that homeobox genes may be downstream from other, more global, master regulatory genes. Indeed, *Prox1* can only reprogram a vascular EC to take on the phenotype of lymphatic endothelium (Petrova et al. 2002). It cannot so reprogram other cell types.

3. Little is known about how homeobox genes implicated in angiogenesis and vascular remodeling exert their effects at the molecular level. However, it is clear that at least a subset of them appear to function by controlling the differentiation, proliferation, and/or migration of VSMCs and ECs. The mechanism behind these phenotypic changes must be the activation and repression of specific batteries of downstream genes. Because few downstream genes from homeobox genes are known, one of the most fertile areas of research for homeobox gene research is the identification of their downstream targets and the elucidation of the mechanisms by which homeobox genes regulate the expression of these target genes and these target genes in turn lead to the phenotypic changes observed. In the near future, it is likely that cDNA microarray technology will provide an excellent tool for identifying the global changes in gene expression occurring in response to homeobox gene expression in vascular cells.

Given their importance in cell-cycle control, cell migration, and cell adhesion, it is likely that many more homeobox genes will be implicated in the regulation of vascular remodeling and angiogenesis. The identification of the specific homeobox genes involved in these processes, their downstream target genes, and the cell-signaling pathways activated and repressed by homeobox gene expression in vascular ECs and VSMCs will result in a better understanding of the basic cellular mechanisms by which the vascular system is remodeled in response to physiologic signals, tumors, or other stimuli. Such understanding has the potential to lead to the development of therapies that block tumor angiogenesis and lymphatic metastasis, reverse atherosclerosis, prevent restenosis after angioplasty, improve wound healing, and reverse lymphedema.

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The Homeobox Gene *Gax* Inhibits Angiogenesis through Inhibition of Nuclear Factor-κB–Dependent Endothelial Cell Gene Expression

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Abstract

The growth and metastasis of tumors are heavily dependent on angiogenesis, but much of the transcriptional regulation of vascular endothelial cell gene expression responsible for angiogenesis remains to be elucidated. The homeobox gene *Gax* is expressed in vascular endothelial cells and inhibits proliferation and tube formation *in vitro*. We hypothesized that *Gax* is a negative transcriptional regulator of the endothelial cell angiogenic phenotype and studied its regulation and activity in vascular endothelial cells. Several proangiogenic factors caused a rapid down-regulation of *Gax* mRNA in human vascular endothelial cells, as did conditioned media from breast cancer cell lines. In addition, *Gax* expression using a replication-deficient adenoviral vector inhibited human umbilical vein endothelial cell migration toward proangiogenic factors *in vitro* and inhibited angiogenesis *in vivo* in Matrigel plugs. To identify putative downstream targets of *Gax*, we examined changes in global gene expression in endothelial cells due to *Gax* activity. *Gax* expression resulted in changes in global gene expression consistent with a quiescent, nonangiogenic phenotype, with increased expression of cyclin kinase inhibitors and decreased expression of genes implicated in endothelial cell activation and angiogenesis. Further analysis revealed that *Gax* down-regulated numerous nuclear factor-κB (NF-κB) target genes and decreased the binding of NF-κB to its target sequence in electrophoretic mobility shift assays. To our knowledge, *Gax* is the first homeobox gene described that inhibits NF-κB activity in vascular endothelial cells. Because NF-κB has been implicated in endothelial cell activation and angiogenesis, the down-regulation of NF-κB–dependent genes by *Gax* suggests one potential mechanism by which *Gax* inhibits the angiogenic phenotype. (Cancer Res 2005; 65(4): 1414-24)

Introduction

The process of angiogenesis, critical in both normal physiology and in disease states such as cancer and inflammatory diseases, is normally tightly regulated by a balance between pro- and antiangiogenic factors, known as the "angiogenic balance" (1). Tumors manipulate their microenvironment and parasitize the host by secreting factors that induce angiogenesis, tipping the angiogenic balance toward a proangiogenic state. The primary target of tumor-secreted proangiogenic factors is the vascular endothelial cell, which becomes "activated" and undergoes distinct changes in phenotype and gene expression. These changes include activation of proteolytic enzymes to degrade basement membrane, sprouting, proliferation, tube formation, and production of extracellular matrix (2, 3). Although the endothelial cell receptors and signaling pathways activated by proangiogenic factors such as vascular endothelial growth factor (VEGF; ref. 4) have been extensively studied, less is known about the molecular biology of the downstream transcription factors activated by these factors. Nuclear transcription factors likely integrate these upstream signals, activating and repressing downstream batteries of genes, to produce an angiogenic global gene expression profile, resulting in the angiogenic phenotype. Consequently, understanding the transcriptional mechanisms by which endothelial cells become activated is likely to suggest new therapeutic strategies for inhibiting this process at a very distal point in its signaling cascade, with potential applications in the antiangiogenic therapy of cancer.

Because of their ubiquitous role as regulators of cellular differentiation and body plan formation during embryogenesis, as well as oncogenes and tumor suppressors in various human cancers (5, 6), it is not surprising that homeobox genes have been implicated in regulating the phenotypic changes that endothelial cells undergo during angiogenesis (7). In particular, one diverged homeobox gene, *Gax* (whose mouse homologue is known as *Meox-2*), has several characteristics that suggest that it may play an important role as an inhibitor of the endothelial cell phenotypic changes that occur in response to stimulation by proangiogenic or proinflammatory factors (8-11). Originally isolated from vascular smooth muscle (8) and widely expressed in mesoderm and muscle precursors in the embryo (12, 13), in the adult *Gax* expression is mostly restricted to the cardiovascular system and kidney (8, 13). In vascular smooth muscle cells, *Gax* expression is down-regulated by mitogens and up-regulated by growth arrest signals (8, 14). Consistent with this observation, *Gax* expression induces G_1 cell cycle arrest (10) and inhibits vascular smooth muscle cell migration, modulating integrin expression (11). *In vivo*, *Gax* expression in arteries inhibits proliferative restenosis of the arterial lumen after injury (10). Recently, we have reported that *Gax* is also expressed in endothelial cells, in which its expression inhibits endothelial cell proliferation (15) and strongly inhibits VEGF-induced endothelial cell tube formation on reconstituted basement membrane *in vitro* (15), suggesting that *Gax* may be an inhibitor of the activated, angiogenic phenotype.

Until now, we had not identified potential mechanisms by which *Gax* might accomplish its inhibition of endothelial cell activation, other than a general cell cycle arrest due to induction of p21 (10, 15). In this report, we now describe how *Gax* expression is regulated in endothelial cells by proangiogenic and proinflammatory factors and how its expression in endothelial

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cells can block angiogenesis *in vivo*. Finally, we present evidence that *Gax* inhibits nuclear factor-κB (NF-κB) activity in endothelial cells. Given that there is now considerable evidence that activation of NF-κB activity in endothelial cells is proangiogenic (16–22), this interaction between a homeobox gene and NF-κB represents one potential mechanism by which *Gax* expression may inhibit angiogenesis. This interaction, to our knowledge the first described in endothelial cells, may represent a new mechanism by which homeobox genes can interact with intracellular signaling pathways in endothelial cells and thereby inhibit tumor-induced angiogenesis.

Materials and Methods

Cell Lines and Expression Constructs

Human umbilical vein endothelial cells (HUVEC) and EGM-2 medium were obtained from BioWhittaker (Walkersville, MD) and HUVECs cultured according to the manufacturer's instructions. Human microvascular endothelial cells (HMEC)-1 cells were obtained from the Centers for Disease Control and were cultured as described (23). Breast cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA) and cultured according to instructions. Conditioned medium was obtained by incubating them in serum-free medium for 24 hours.

The cloning of the *Gax* cDNA into the mammalian expression vector pCGN to produce pCGN-*Gax* and the construction of replication-deficient adenoviral vectors expressing the rat and human homologues of *Gax* (Ad.h*Gax* and Ad.r*Gax*, respectively) conjugated to the α-hemagglutinin epitope have been described (10). The control replication-deficient adenoviral vector expressing green fluorescent protein (Ad.*GFP*) was a kind gift of Dr. Daniel Medina (The Cancer Institute of New Jersey, New Brunswick, NJ). An adenoviral construct expressing a form of Akt (T308A, S473A, adenoviral construct designated Ad.DN.Akt) that functions as a dominant negative (24) was kindly provided by Dr. Kenneth Walsh (Boston University, Boston, MA). Expression of *Gax* protein was verified as previously described (13) by Western blot using antihemagglutinin antibody and anti-*Gax* antibodies (not shown). Transfections of HUVECs with pCGN-*Gax* were carried out using Trans-IT Jurkat transfection reagent (Mirus Bio Corporation, Madison, WI) according to a modification of the manufacturer's instructions.

Real-time Quantitative Reverse Transcription–PCR

After treatment as described individually for each experiment, total RNA was isolated from endothelial cells using a spin column with on-column DNase digestion to remove contaminating genomic DNA (RNAeasy, Qiagen, Valencia, CA). First-strand synthesis was done on the total RNA using oligo(dT) primers (SuperScript kit, Invitrogen, Carlsbad, CA), and then message levels for *Gax* and other genes determined by real-time quantitative reverse transcription–PCR (RT-PCR) using TaqMan probes (25). Quantitative RT-PCR was carried out using a Cepheid SmartCycler thermocycler, with the associated SmartCycler v2.0 software used to analyze the data and determine the threshold count (C_t).

Primer and probe sets for each gene were designed using the MacVector 7.2 software package (Accelrys, San Diego, CA). The fluorophore used was 6-carboxyfluorescein (6-FAM), and the quencher was Black Hole Quencher-1 (BHQ-1, Biosearch Technologies, Novato, CA). Sequences of the primers and probes were as follows: *Gax* 5′-TCA GAA GTC AAC AGC AAA CCC AG-3′ (forward), 5′-CCA GTT CCT TTT CCC GAG-3′ (reverse), 5′-(6-FAM)-TGG TTC CAA AAC AGG CGG ATG-3′-(BHQ1; TaqMan probe), amplicon = 238 bp; E-selectin: 5′-CTC TGA CAG AAG AAG CCA AG-3′ (forward), 5′-ACT TGA GTC CAC TGA AGT CA-3′ (reverse), 5′-(6-FAM)-CCA CGC AGT CCT CAT CTT TTT G-3′ (BHQ1; TaqMan probe), amplicon = 255 bp; vascular cell adhesion molecule-1 (VCAM-1): 5′-ATG ACA TGC TTG AGC CAG G-3′ (forward), 5′-GTG TCT CCT TCT TTG ACA CT-3′ (reverse), 5′-(6-FAM)-CAC TTC CTT TCT GCT TCT TCC AGC-3′ (BHQ1; TaqMan probe), amplicon = 260 bp; intercellular adhesion molecule-1 (ICAM-1): 5′-TAT GGC AAC GAC TCC TTC T-3′ (forward), 5′-CAT TCA GCG TCA CCT TGG-3′ (reverse), 5′-(6-FAM)-CCT TCT GAG ACC TCT GGC TTC G-3′-(BHQ1, TaqMan probe), amplicon = 238 bp; GRO-α: 5′-CAA GAA CAT CCA AAG TGT GAA CG-3′ (forward), 5′-(6-FAM)-AGG AAC AGC CAC CAG TGA GC-3′ (reverse), 5′-CGC CCA AAC CGA AGT CAT AGC-3′-(BHQ-1; TaqMan probe), amplicon=200 bp. Sequences of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primer and probe set were 5′-ACA ACT TTG GTA TCG TGG AAG-3′

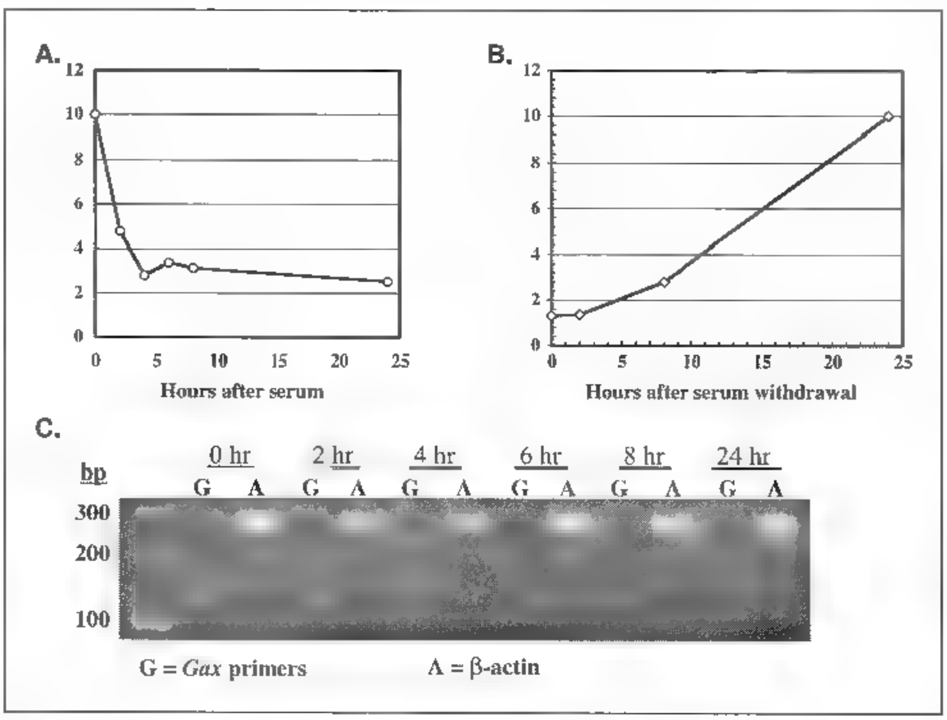

Figure 1. *Gax* expression is down-regulated induced in HUVECs by serum and up-regulated when serum is withdrawn. Using real-time quantitative RT-PCR, *Gax* levels were measured in quiescent HUVECs stimulated with serum and randomly cycling HUVECs placed in low-serum medium. *Gax* levels were normalized to β-actin. For this experiment alone, primers for *Gax* and β-actin previously described were used (15). Similar results were obtained with the primer/probe combination described in Materials and Methods. *A*, *Gax* is down-regulated by serum. *B*, *Gax* is up-regulated by serum withdrawal. *C*, PCR gel of the experiment in *A*. Units are arbitrary.

(forward), 5′-CAG ATG AGG CAG GGA TGA TGT TC-3′ (reverse), and 5′-(6-FAM)-ACC CAG AAG ACT GTG GAT GG-3′-(BHQ1; TaqMan probe), amplicon = 138 bp. For some experiments (Fig. 1), a set of primers for human *Gax* and β-actin previously described were used (15), along with SYBr Green to monitor the PCR reaction.

Real-time PCR cycles started with an initial 1.5-minute denaturation step at 95°C, followed by 30 to 40 cycles of denaturation at 95°C for 10 seconds; annealing at 50°C (VCAM-1), 52°C (E-selectin, ICAM-1), and 56°C (*Gax*, GAPDH, p21, Gro-α) for 20 seconds; and extension at 72C for 30 seconds. Each sample was run in triplicate and C_t determined for the target gene. For all reactions, negative controls were run with no template present, and random RNA preparations were also subjected to sham quantitative RT-PCR (no reverse transcriptase) to verify lack of genomic DNA contamination. To correct for differences in RNA quality and quantity between samples, target gene levels were normalized to corresponding GAPDH message levels using the $\Delta\Delta C_t$ method (26), as described previously (27, 28).

Migration Assays

Before each experiment, cell culture membranes and flasks were coated with sterile 0.1% gelatin in PBS. HUVECs were infected with adenoviral vectors for 16 hours before 5×10^4 cells per well were plated onto 8.0-μm pore size polycarbonate membrane in 24-well plates. Cells were allowed to attach for 1 hour in EGM-2 medium. Once the cells had attached, the medium in the upper chamber was replaced with low-serum medium [which consisted of EGM-2 + 0.1% fetal bovine serum (FBS) lacking VEGF, basic fibroblast growth factor (bFGF), and epidermal growth factor], and the lower chamber with low-serum medium supplemented with either 50 ng/mL VEGF, 50 ng/mL bFGF, 15 ng/mL tumor necrosis factor (TNF), or 10% FBS. VEGF, bFGF, and TNF-α all obtained from R&D Systems (Minneapolis, MN). After 5 hours, the inserts were washed with PBS and the upper surfaces cleaned with a cotton swab to remove any cells that had not migrated. Finally the cells were fixed with Diff-Quik Stain (Dade Behring, Deerfield, IL) and the inserts washed in PBS and photographed for counting. Cells were counted in five high-powered fields per well. Experiments were repeated at least thrice.

In vivo Angiogenesis Assay

In vivo angiogenesis was assayed by the Matrigel plug assay as described previously (24). These experiments were done under a protocol approved by the Institutional Animal Care and Use Committee at University of Medicine and Dentistry of New Jersey-Robert Wood Johnson Medical School. In brief, cold, low growth factor Matrigel (BD PharMingen, San Diego, CA, 500 μL per mouse) containing bFGF 400 ng/mL (R&D Systems), heparin 10 units/mL (Sigma, St. Louis, MO), and 10^8 plaque-forming units of adenoviral expression vector were injected into the flanks of C57BL/6 mice. After 14 days, the mice were euthanized by CO_2 inhalation, and the plugs carefully removed *en bloc* with surrounding connective tissue. Tissue and plugs were fixed in cold acetone and frozen sections cut at 5 μm. Endogenous peroxidase activity was blocked with dilute H_2O_2. Sections were then blocked with 5% bovine serum albumin (BSA) for 15 minutes, washed with PBS, and then incubated with rat anti-mouse CD31 (PECAM) monoclonal antibody (BD PharMingen) in 1% BSA in PBS overnight. Sections were washed with cold PBS twice and incubated with biotinylated mouse anti-rat IgG1, 2a (BD PharMingen) in 1% BSA/PBS. Color was then developed with streptavidin-peroxidase (VectaStain, ABC kit, Vector Laboratories, Burlingame, CA). Sections were counterstained with toluidine blue and vessel counts done as previously described (24, 29). In brief, vascular hotspots were located for each plug near the interface between the plug and surrounding stroma, and blood vessel density estimated as the number of CD31-positive cells per high-powered field. Two sections from each plug were made, at least five high-powered fields per section counted, and the mean ± SE determined for each experimental group. The experiment was repeated twice. Statistical differences were determined by one-way ANOVA using Prism v.4.0 (GraphPad Software, Inc., San Diego, CA), followed by Dunnett's multiple comparison test.

Genome-wide Gene Expression Profiling

We compared global gene expression in control HUVECs transduced with Ad.GFP with that of HUVECs transduced with Ad.r*Gax* or Ad.h*Gax*. Cells were transduced at a multiplicity of infection (MOI) of 100, incubated 24 hours in normal medium, then harvested for total RNA isolation as described above. RNA quality was verified by electrophoresis through formaldehyde-containing agarose gels before use for generating probes. Exogenous *Gax* expression was verified by Western blot (data not shown). Global gene expression was then compared in two separate experiments using the Affymetrix Human Genome U133A GeneChip array set and standard protocols supplied by the manufacturer, with technical assistance from the cDNA Microarray Core Facility of the Cancer Institute of New Jersey. The U133A chip contains probe sets for over 33,000 known genes, along with probes for housekeeping genes for normalization and genomic DNA for evaluation of hybridization quality. Results were analyzed using software provided by the manufacturer and then further analyzed with GeneMAPP (30) to identify signal pathway–dependent changes in gene expression.

Western Blots

Whole cell extracts from TNF-α-treated HUVECs were electrophoresed through 8% SDS-polyacrylamide gels and transferred to polyvinylidene diflouride membranes. The membranes were blocked with PBS plus 5% nonfat dry milk and 0.1% Tween 20 before being incubated with the appropriate dilution of primary antibody (mouse monoclonal anti-VCAM-1 and anti-ICAM-1 and rabbit polyclonal anti-E-selectin, Santa Cruz Biotechnology, Santa Cruz, CA) in blocking solution. Blots were washed with blocking solution and incubated with secondary antibody (goat anti-mouse IgG or goat anti-rabbit IgG; Pierce Biotechnology, Inc., Rockford, IL) and then washed again with blocking solution. Bands were visualized by chemiluminescence using the ECL-Plus reagent (Amersham, Piscataway, NJ).

Flow Cytometry

Cells were harvested after the relevant treatment and resuspended in PBS containing 0.1% sodium azide. Approximately 1×10^5 cells were incubated with FITC-conjugated primary antibody against human E-selectin, VCAM-1, or ICAM-1 (BD Biosciences, San Diego, CA) for 30 minutes on ice. Cells were pelleted and washed twice in PBS/azide before flow analysis on a Beckman-Coulter Cytomics FC500 flow cytometer (Fullerton, CA).

Electrophoretic Mobility Shift Assays

HUVECs were transduced overnight with Ad.GFP or Ad.r*Gax* and then induced with 10 ng/mL TNF-α for 1 hour. Nuclear extracts were prepared with the NE-PER nuclear extraction reagent (Pierce Biotechnology) and incubated with a biotin end-labeled double-stranded oligonucleotide containing the NF-κB consensus sequence (5′-biotin-AGT TGA GGG GAC TTT CCC AGG C-3′; IDT DNA Technologies, Coralville, IA). The binding reactions, containing 6 to 8 μg of nuclear extract protein, buffer [10 mmol/L Tris (pH 7.5), 50 mmol/L KCl, 1 mmol/L DTT], 1 μg of poly(deoxyinosinic-deoxycytidylic acid), 5 μg BSA, and 20 fmol/L of biotin-labeled DNA, were incubated at room temperature for 20 minutes. Competition reactions were done by adding up to 200-fold excess unlabeled double-stranded NF-κB consensus oligonucleotide to the reaction mixture. Other controls included competition with random oligonucleotide (5′-TAG CAT ATG CTA-3′) and an NF-κB site with a point mutation that abolishes DNA binding (5′-CAC AGT TGA GGC CAC TTT CCC AGG C-3′). Reactions were electrophoresed on a 6% acrylamide gel at 100 V for 1 hour in 0.5× Tris-borate-EDTA buffer and then transferred to positively charged nylon membranes. Biotinylated oligonucleotides were detected with streptavidin-linked horseradish peroxidase and the Pierce LightShift kit (Pierce Biotechnology).

Results

Gax Expression Is Rapidly Down-regulated by Mitogens and Proangiogenic Factors in Endothelial Cells

We first wished to determine how *Gax* expression is regulated by growth factors and proangiogenic peptides in endothelial cells.

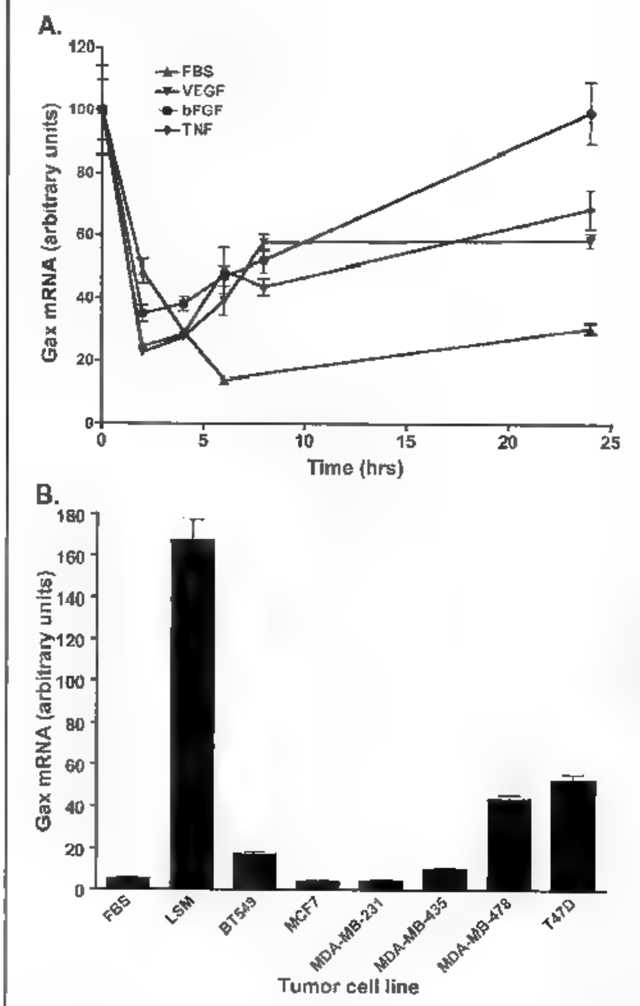

Figure 2. *Gax* down-regulation by mitogens, proinflammatory factors, and tumor-secreted factors. *A*, Mitogens and proangiogenic factors cause rapid down-regulation of Gax expression in endothelial cells. Quiescent HUVECs were treated with either 10% FBS or 10 ng/mL of either $VEGF_{165}$, TNF-α, or bFGF. At various time points, cells were harvested for extraction of total RNA, which was then subjected to quantitative real-time TaqMan RT-PCR with *Gax*- and GAPDH-specific primer/probe sets. (See Materials and Methods for sequences and details.) *B*, down-regulation of *Gax* expression in endothelial cells by conditioned medium from tumor cell lines. Quiescent HUVECs were treated with either low-serum medium, 10% FBS, or 10% conditioned medium from the indicated breast cancer cell lines. Cells were harvested 4 hours after stimulation, total RNA harvested, and real time quantitative RT-PCR done. All *Gax* mRNA levels were normalized to GAPDH expression, and units are arbitrary.

HUVECs made quiescent by incubation for 24 hours in 0.1% FBS were stimulated with 10% FBS plus 5 ng/mL VEGF. *Gax* mRNA was rapidly down-regulated by 5-fold within 4 hours and slowly returned to basal over 24 to 48 hours (Fig. 1*A* and *C*). Conversely, when sparsely plated randomly cycling HUVECs were placed in medium containing 0.1% serum, *Gax* was up-regulated nearly 10-fold within 24 hours (Fig. 1*B*). Quiescent HUVECs were then stimulated with proangiogenic or proinflammatory factors, including bFGF, VEGF, and TNF-α. *Gax* was rapidly down-regulated with a similar time course (Fig. 2*A*). Similar results were observed in HMEC-1 cells (23), an immortalized human microvascular endothelial cell line (data not shown). Finally, conditioned medium from several breast cancer cell lines was used to stimulate quiescent HUVECs for 4 hours. The cell lines varied considerably in their ability to down-regulate *Gax*, but all of them down-regulated *Gax* expression at least 3-fold, and some by as much as 20-fold (Fig. 2*B*), suggesting that tumor-secreted proangiogenic factors also down regulate *Gax* expression.

Gax Expression Inhibits Endothelial Cell Migration toward Proangiogenic Factors

Migration of endothelial cells through the basement membrane and into the surrounding stroma in response to proangiogenic stimuli is a critical step in tumor-induced angiogenesis. We therefore tested the ability of *Gax* to inhibit endothelial cell migration toward proangiogenic factors. HUVECs were transduced with Ad.r*Gax* or Ad.h*Gax* at varying MOI and incubated overnight. Viable cells (10^5 per well) were plated in six-well plates with inserts containing 8-μm polycarbonate filters and their migration toward angiogenic factor–containing media in the lower chamber

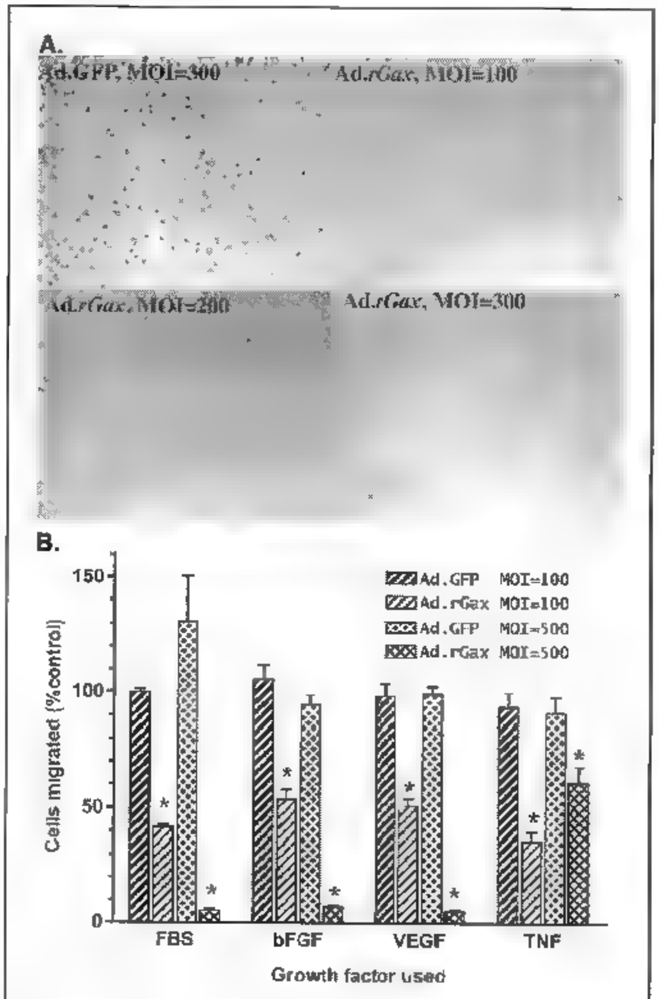

Figure 3. *Gax* inhibits HUVEC migration toward serum. HUVECs were transduced with varying MOIs of either Ad.*GFP* or Ad.r*Gax* and their migration toward various growth factors and proangiogenic factors determined (see Materials and Methods). *Gax* inhibits HUVECs migrating toward (*A*) *FBS* and (*B*) *FBS*, *bFGF*, $VEGF_{165}$, and *TNF-α*. Results are expressed relative to control HUVECs not transduced with any virus. Results were analyzed by one-way ANOVA. *, $P < 0.01$. Similar results were obtained with Ad.h*Gax* (data not shown).

measured. Ad.r*Gax* strongly inhibited the migration of HUVECs toward serum, VEGF, bFGF, and TNF-α (Fig. 3), as did Ad.h*Gax* (data not shown). Both homologues also inhibited migration of HMEC-1 cells toward bFGF and VEGF (data not shown).

Gax Expression Inhibits *In vivo* Angiogenesis

Matrigel containing proangiogenic factors, when implanted s.c. in mice, can stimulate the ingrowth of blood vessels into the Matrigel plug from the surrounding tissue, allowing *in vivo* tumor cell-free estimates of angiogenesis (24). Moreover, adenoviral vectors diluted in Matrigel implanted as s.c. plugs can serve as reservoirs to transduce endothelial cells invading the plug and drive expression of exogenous genes, producing effects on *in vivo* angiogenesis (31). We therefore used Matrigel plugs to test whether exogenously driven *Gax* expression can inhibit angiogenesis *in vivo*, using methodology previously described (24). Matrigel plugs containing bFGF and either Ad.GFP, Ad.h*Gax*, or Ad.r*Gax* (see Materials and Methods) were injected s.c. into C57BL/6 mice (n = 8 per experimental group). As a positive control for inhibition of angiogenesis *in vivo* by a viral vector, we used an additional adenoviral construct expressing a form of Akt (T308A, S473A, adenoviral construct designated Ad.DN.Akt) that functions in a dominant-negative fashion (24) and has previously been used in the Matrigel plug assay to show that inhibition of Akt signaling inhibits angiogenesis *in vivo* (24). As another control, to verify that adenovirus itself does not significantly alter *in vivo* angiogenesis as measured by this assay, plugs containing only bFGF were also examined. Adenoviral vectors expressing *Gax* expression were observed to inhibit the neovascularization of the plugs with a potency slightly less than what was observed for the Ad.DN Akt construct (Fig. 4), and the Ad.DN.Akt construct inhibited neovascularization with a potency similar to what has previously been reported (24).

Gax Expression Down-regulates the Expression of NF-κB Target Genes

Next, in order to attempt to identify downstream targets and signaling pathways regulated by *Gax* expression, we determined differences in global gene expression between control HUVECs infected with Ad.GFP with HUVECs infected with Ad.r*Gax* or Ad.h*Gax*. Cells were infected at an MOI = 100, incubated 24 hours

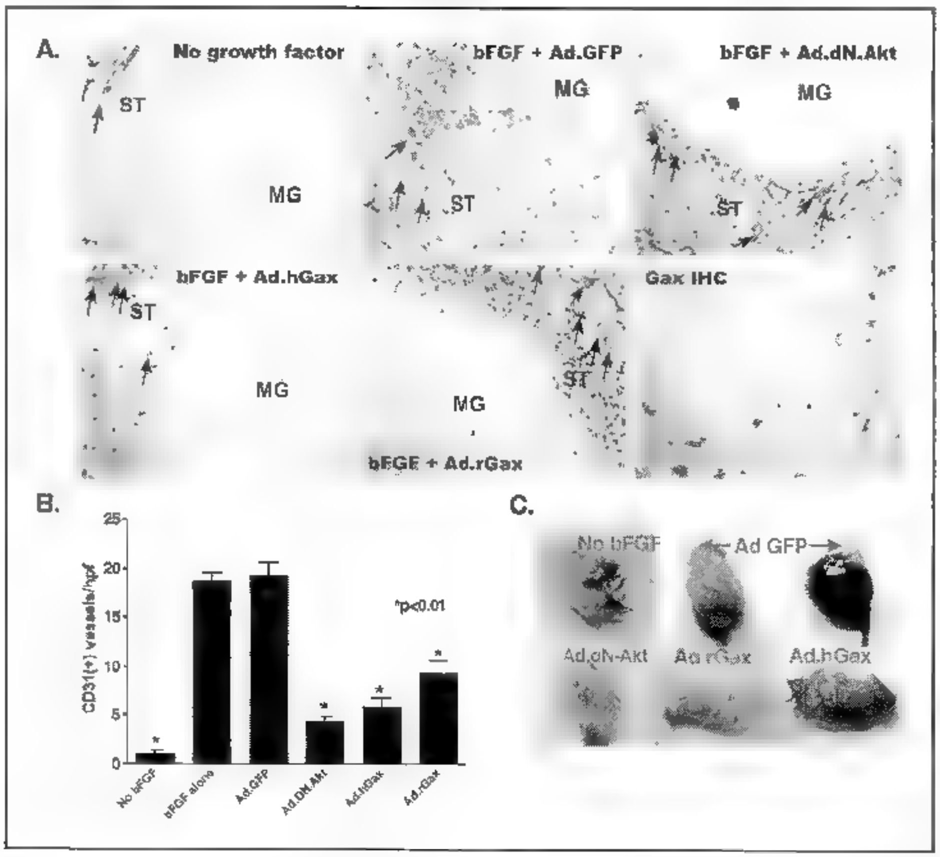

Figure 4. Effect of *Gax* expression on angiogenesis in Matrigel plugs. Matrigel plugs (500 μL each) containing 400 ng/mL bFGF and the indicated viral constructs at 10^8 plaque-forming units per plug were implanted s.c. in the flanks of C57BL6 mice. Plugs were harvested after 14 days incubation for immunohistochemistry using CD31 antibodies and determination of CD31 positive cells per high powered (400x) field (see *Materials and Methods* and *Results* for details). *MG*, Matrigel plug. *ST*, stroma surrounding the plug. *Arrows*, examples of CD31-positive blood vessels. *A*, *Gax* inhibits in vivo angiogenesis. Plugs with either no growth factor or bFGF plus Ad.GFP, Ad.dN.Akt, Ad.h*Gax*, or Ad.r*Gax* were implanted into the flanks of C57BL/6 mice (see Materials and Methods for details and concentrations). After 14 days, the mice were euthanized and the plugs harvested for immunohistochemistry with CD31. Immunohistochemistry using anti-*Gax* antibodies according to previously described methods (13) was done on a representative plug into which Ad.rGax had been introduced to show that the construct is transducing the cells within the plug (*lower right hand corner*). *B*, vessel counts. *Columns*, means; *bars*, SE. Statistical differences determined with one-way ANOVA, $P < 0.0001$ for the overall. The vessel counts were statistically significantly different from control (Ad.GFP group) for Ad.DN.Akt ($P = 0.013$), Ad.h*Gax* ($P = 0.008$), and Ad.r*Gax* ($P = 0.028$). *C*, gross photographs of selected plugs. Note the hemorrhage into one of the Ad.GFP plugs and the lack of vessels on the capsule of the Ad.*Gax* and Ad.dN.Akt plugs.

Table 1. Genes regulated by *Gax* expression

Genbank no	Gene	Function	Fold change	*P*
Up-regulated Genes				
L37882	Frizzled homologue 2 (FZD2)	Signal transduction	30.4	<0.0001
NM_025151	Rab coupling protein (RCP)	Signal transduction	30.1	0.0026
AI678679	Bone morphogenetic protein receptor, type IA (BMPR1A, ALK3)	Signal transduction	27.9	0.0015
N74607	Aquaporin 3 (AQP3)	Transport	19.9	0.0011
AI983115	Class I cytokine receptor	Signal transduction	12.1	<0.0001
NM_002276	Keratin 19 (KRT19)	Structural protein	9.2	<0.0001
NM_004727	Solute carrier family 24 member 1 (SLC24A1)	Ion transport	9.2	0.0007
NM_004585	Retinoic acid receptor responder (tazarotene induced) 3	Cell growth inhibition	8.5	0.0077
K01228	Proα 1 (I) chain of type 1 procollagen	Structural protein	6.4	0.0001
NM_000361	*Thrombomodulin (THBD)*	*Coagulation*	*5.5*	*0.0006*
NM_006931	Solute carrier family 2 (facilitated glucose transporter), member 3 (SLC2A3)	Biosynthesis/metabolism	5.3	0.0000
NM_000850	Glutathione *S*-transferase M4 (GSTM4)	Biosynthesis/metabolism	4.9	0.0009
NM_002064	Glutaredoxin (thioltransferase; GLRX)	Biosynthesis/metabolism	4.9	0.0001
AF162769	Thioltransferase	Biosynthesis/metabolism	4.6	<0.0001
NM_002166	*Inhibitor of DNA binding 2 (ID2)*	*Transcriptional regulation*	*4.6*	*<0.0001*
NM_017436	α1,4-galactosyltransferase; 4-*N*-acetylglucosaminyltransferase (A14GALT)	Biosynthesis/metabolism	4.3	0.0003
NM_005904	MAD (mothers against decapentaplegic) homologue 7 (MADH7)	Signal transduction	4.3	0.0006
NM_000170	Glycine dehydrogenase (GLDC)	Biosynthesis/metabolism	4.0	0.0003
NM_002222	Inositol 1,4,5-triphosphate receptor, type 1 (ITPR1)	Signal transduction	4.0	0.0000
NM_000229	Lecithin-cholesterol acyltransferase (LCAT)	Biosynthesis/metabolism	4.0	0.0002
M25915	Complement cytolysis inhibitor (CLI)	Complement activation	3.7	<0.0001
AF326591	Fenestrated-endothelial linked structure protein (FELS)	Structural protein	3.7	<0.0001
NM_001666	GTPase activating protein 4 (ARHGAP4)	Signal transduction	3.7	<0.0001
NM_006456	Sialyltransferase (STHM)	Biosynthesis/metabolism	3.7	0.0001
NM_000050	Argininosuccinate synthetase (ASS)	Biosynthesis/metabolism	3.7	<0.0001
AF035620	BRCA1-associated protein 2 (BRAP2)	Biosynthesis/metabolism	3.5	0.0002
M25915	Cytolysis inhibitor (CLI)	Complement activation	3.5	<0.0001
NM_006736	Heat shock protein, neuronal DNAJ-like 1 (HSJ1)	Stress response	3.5	<0.0001
NM_000693	Aldehyde dehydrogenase 1 family, member A3 (ALDH1A3)	Biosynthesis/metabolism	3.5	<0.0001
NM_000213	*Integrin subunit, 4 (ITGB4)*	*Cell adhesion*	*3.5*	*0.0001*
NM_003043	Solute carrier family 6, member 6 (SLC6A6)	Transport	3.5	0.0001
AF010126	Breast cancer-specific protein 1 (BCSG1)	Unknown	3.2	0.0002
NM_005345	Heat shock 70kD protein 1A (HSPA1A)	Stress response	3.2	<0.0001
NM_006254	Protein kinase C, δ (PRKCD)	Signal transduction	3.0	0.0001
NM_000603	Nitric oxide synthase 3 (endothelial cell; NOS3)	Biosynthesis/metabolism	3.0	<0.0001
U20498	Cyclin-dependent kinase inhibitor p19INK4D	Cell cycle	2.5	0.0004
NM_001147	*Angiopoietin 2 (ANGPT2)*	*Cell growth/chemotaxis*	*2.2*	*0.0023*
N33167	Cyclin-dependent kinase inhibitor 1C (p57, Kip2)	Cell cycle	2.1	0.0065
Down-regulated genes				
NM_002167	*Inhibitor of DNA binding 3 (ID3)*	*Transcriptional regulation*	*2.0*	*0.0081*
D13889	*Inhibitor of DNA binding 1 (ID1)*	*Transcriptional regulation*	*2.1*	*0.0052*
NM_001546	*Inhibitor of DNA binding 4 (ID4)*	*Transcriptional regulation*	*2.1*	*0.0056*
M60278	***Heparin-binding epidermal growth factor–like growth factor***	***Cell growth/chemotaxis***	***−2.1***	***0.0056***
NM_001955	***Endothelin 1 (EDN1)***	***Cell growth/chemotaxis***	***2.5***	***0.0007***
NM_000201	***Intercellular adhesion molecule 1 (ICAM1)***	***Signal transduction***	***−2.5***	***0.0059***
NM_004995	*Matrix metalloproteinase 14*	*Proteolysis*	*−2.7*	*0.0002*
NM_002006	*Fibroblast growth factor 2 (basic; FGF2)*	*Cell growth/chemotaxis*	*2.8*	*0.0244*
NM_004428	*Ephrin-A1 (EFNA1)*	*Cell growth/chemotaxis*	*−3.0*	*0.0042*
AF021834	Tissue factor pathway inhibitor β (TFPIβ)	Coagulation	−3.0	0.0007

(Continued on the following page)

Table 1. Genes regulated by *Gax* expression (Cont'd)

Genbank no	Gene	Function	Fold change	*P*
NM_016931	NADPH oxidase 4 (NOX4)	Biosynthesis/metabolism	−3.2	0.0029
NM_021106	Regulator of G-protein signaling 3 (RGS3)	Signal transduction	3.5	0.0059
NM_002130	3-Hydroxy-3-methylglutaryl-coenzyme A synthase 1 (soluble; HMGCS1)	Biosynthesis/metabolism	3.5	0.0008
NM_001146	*Angiopoietin 1 (ANGPT1)*	*Cell growth/chemotaxis*	*3.9*	*0.0012*
NM_005658	***TNF receptor–associated factor 1***	***Signal transduction***	***−4.0***	***0.0086***
NM_001721	BMX nonreceptor tyrosine kinase (BMX), mRNA	Signal transduction	4.3	0.0007
NM_006226	Phospholipase C, epsilon (PLCE)	Signal transduction	−4.3	0.0012
NM_006823	Protein kinase (cyclic AMP–dependent, catalytic) inhibitor α (PKIA)	Signal transduction	−4.3	0.0002
NM_002425	*Matrix metalloproteinase 10*	*Proteolysis*	*4.4*	*0.0002*
NM_016315	CED-6 protein (CED-6)	Vesicle-mediated transport	−4.6	0.0059
NM_000600	***Interleukin 6 (IFN, β 2; IL6)***	***Cell growth/chemotaxis***	***−4.6***	***0.0020***
M68874	Phosphatidylcholine 2-acylhydrolase (cPLA2)	Signal transduction	4.9	0.0007
U58111	***Vascular endothelial growth factor C (VEGF-C)***	***Cell growth/chemotaxis***	***5.3***	***0.0020***
NM_003326	**TNF (ligand) superfamily, member 4 (TNFSF4)**	**Signal transduction**	**5.7**	**0.0021**
AB040875	Cystine-glutamate exchanger	Biosynthesis/metabolism	6.1	0.0012
NM_006290	***TNF-α-induced protein 3 (A20, TNFAIP3)***	***Apoptosis***	***6.4***	***0.0009***
S69738	**Monocyte chemotactic protein human (MCP-1)**	**Cell growth/chemotaxis**	**−6.5**	**0.0303**
NM_012242	Dickkopf homologue 1 (DKK1)	Signal transduction	8.0	0.0002
NM_002852	**Pentaxin-related gene, rapidly induced by IL-1 β (PTX3)**	**Immune response**	**−9.2**	**0.0142**
L07555	**Early activation antigen CD69**	**Signal transduction**	**10.6**	**0.0042**
NM_001078	*Vascular cell adhesion molecule 1 (VCAM1)*	*Cell adhesion*	*−13.0*	*0.0303*
NM_002993	**Granulocyte chemotactic protein 2**	**Cell growth/chemotaxis**	**17.5**	**0.0059**
NM_012252	Transcription factor endothelial cell	Transcriptional regulation	18.5	0.0302
NM_000963	Prostaglandin-endoperoxide synthase 2	Biosynthesis/metabolism	26.0	0.0303
NM_001993	***Coagulation factor III (thromboplastin, tissue factor)***	***Coagulation***	***−39.4***	***0.0022***
NM_000450	***E-selectin (SELE)***	***Cell adhesion***	***62.6***	***0.0142***
M57731	***Chemokine (C-X-C motif) ligand 2 (CXCL2, GRO-)***	***Cell growth/chemotaxis***	***−79.6***	***0.0007***
NM_002090	***Chemokine (C-X-C motif) ligand 3 (CXCL3)***	***Cell growth/chemotaxis***	***119.9***	***0.0029***
NM_000584	***Interleukin 8 (IL-8)***	***Immune response***	***181.3***	***0.0142***
NM_004591	***Chemokine (C-C motif) ligand 20 (CCL20)***	***Cell growth/chemotaxis***	***237.6***	***0.0376***
NM_001511	***Melanoma growth stimulating activity, α/GRO-1/GRO-α (CXCL1)***	***Cell growth/chemotaxis***	***238.9***	***0.0059***

NOTE: Boldface, genes induced by NF-κB activity; italicized, genes involved in regulating angiogenesis.

in normal media, then harvested for total RNA isolation. Global gene expression was compared in two separate experiments using the Affymetrix Human Genome U133A GeneChip array set (see Materials and Methods). We observed 127 probe sets corresponding to known genes showing greater than 2-fold up-regulation and 115 showing greater than 2-fold down-regulation. Differences in gene expression between controls and *Gax*-transduced cells ranged from up-regulation by approximately 30-fold to down-regulation by 239-fold. This pattern was similar in endothelial cells transduced by Ad.h*Gax*, although the magnitude of changes in gene expression tended to be smaller (data not shown). We report here only probe sets that represent known genes that were either up- or down-regulated by at least 2.5-fold, with the addition of a few genes regulated <2.5-fold selected because they are either involved in angiogenesis, regulated by NF-κB, or both (Table 1).

Consistent with the hypothesis that *Gax* inhibits endothelial cell activation, *Gax* strongly down-regulated several CXC chemokines (Table 1). Most strongly down-regulated of all was GRO-α (CXCL1), a CXC chemokine and a growth factor for melanoma that has also been implicated in promoting angiogenesis (32). *Gax* also down-regulated cell adhesion molecules known to be up-regulated in endothelial cells during activation and angiogenesis, including VCAM-1, ICAM-1, and E-selectin (33), all of whose down-regulation we have confirmed using real-time quantitative RT-PCR, Western blot, and flow cytometry (Fig. 5). Moreover, *Gax* inhibited both the basal and TNF-α-induced up-regulation of ICAM-1, VCAM-1, and E-selectin proteins (Fig. 5*C* and *D*, and not shown). The pattern of down-regulation of these adhesion molecules, which are normally up-regulated during endothelial cell activation and angiogenesis, coupled with the down-regulation of CXC chemokines, suggested the inhibition of genes normally induced by TNF-α, which in turn suggested the possibility that *Gax* may inhibit NF-κB activity. Indeed, when our data was analyzed using GeneMAPP (30) to look for patterns of signal-dependent gene regulation, numerous NF-κB-dependent genes were identified (Table 1). Western blot analysis showed no difference between untransduced endothelial cells and cells transduced with Ad.GFP in either the

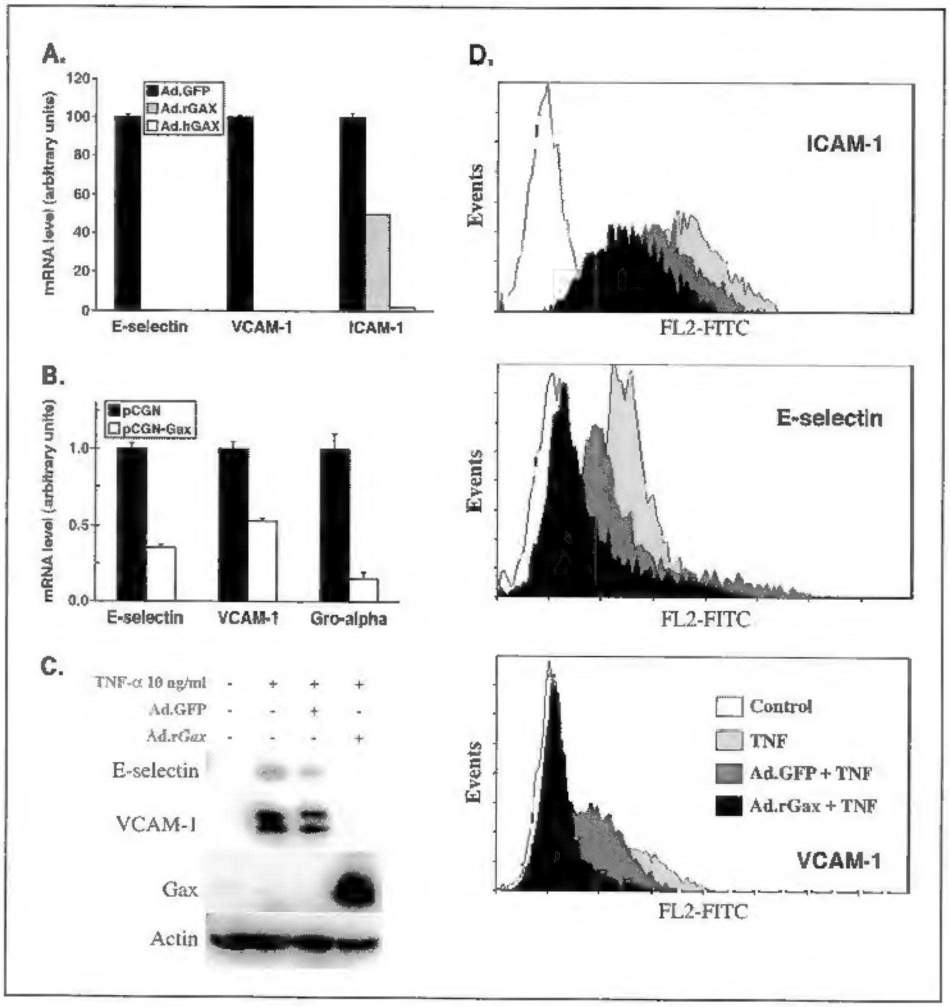

Figure 5. Effect of *Gax* expression on the level of E-selectin, VCAM-1, and ICAM-1. *A, Gax* down-regulates cell adhesion molecule mRNAs in HUVECs. HUVECs were transduced with Ad.GFP, Ad.h*Gax*, or Ad.r*Gax*, incubated for 24 hours in normal growth medium, then harvested for total RNA isolation. Total RNA was then subjected to quantitative real time RT-PCR using TaqMan primers and probes specific for each gene and the results normalized to GAPDH. A very strong down-regulation of E-selectin, VCAM-1, and ICAM-1 message level was observed. *B, Gax* down-regulates NF-κB-dependent genes using nonviral transduction. To rule out artifacts from GFP expression, HUVECs were transfected with pCGN-Gax or pCGN empty vector and then incubated overnight in growth medium. Cells were then harvested for total RNA, which was subjected to real time quantitative RT-PCR as described in Materials and Methods. Despite the lower transfection efficiency of liposomal-mediated methods, a strong down-regulation of NF-κB-dependent genes was observed compared with the empty vector. Units are arbitrary for (*A*) and (*B, C*). *C, Gax* down-regulates HUVEC expression of cell adhesion molecules. HUVECs were transduced with Ad.r*Gax* or Ad.GFP and then incubated overnight, after which they were stimulated with 10 ng/mL TNF-α for 4 hours. Cells were harvested for total protein and subjected to Western blot with appropriate antibodies. Expression of *Gax* from the adenoviral vector was verified by Western blot with antibodies against *Gax* as previously described (13). *Gax* also down-regulated ICAM-1 (not shown). *D, Gax* down-regulates cell surface expression of ICAM-1, E-selectin, and VCAM-1. HUVECs transduced overnight with either Ad.GFP or Ad.r*Gax* at an MOI = 100 were stimulated with TNF-α 10 ng/mL for 4 hours and then harvested for flow cytometry using appropriate antibodies (see Materials and Methods). Ad.r*Gax* blocked the expression of VCAM-1, E-selectin, and ICAM-1.

TNF-α-induced expression of VCAM-1 or E-selectin (Fig. 5*C*) or the basal level of VCAM-1, ICAM-1, or E-selectin protein (not shown), and only slight differences by flow cytometry (Fig. 5*D*), suggesting that our result is not an artifact of our use of Ad.GFP as a control in the initial gene expression profiling experiment. Further supporting this conclusion is our observation by quantitative real time RT-PCR that (1) there was no difference between untransduced HUVECs and those transduced with Ad.GFP in the expression of E-selectin, ICAM-1, VCAM-1, Gro-α, VEGF-C, bFGF, $p21^{CIP1/WAF1}$, and a variety of other genes identified in Table 1 as being regulated by *Gax* (data not shown); and (2) that the same result was obtained for Gro-α, E-selectin, and VCAM-1 using nonviral means of transducing the HUVECs in which no GFP-containing vectors were used (Fig. 5*B*).

In contrast, the genes up-regulated by *Gax* did not fall into any signal-dependent patterns as striking as the genes down-regulated by *Gax* (Table 1). However, there were still results that might suggest specific pathways up-regulated by *Gax*. First, there was a strong up-regulation of ALK3 (bone morphogenetic receptor 1a; 34). Although it is known that ALK1 activates endothelial cells through a SMAD1/5 pathway and ALK5 inhibits endothelial cell activation through a SMAD2/3 pathway (35), it is not known what role ALK3 plays in regulating endothelial cell phenotype. Second, we observed the up-regulation of three CDK inhibitors, $p19^{INK4D}$, $p57^{Kip2}$, and $p21^{WAF1/CIP1}$ (10, 36, 37), consistent with a role in promoting cell cycle arrest and the quiescent phenotype. Finally, *Frizzled-2* was strongly up-regulated. Little is known about the potential role of *Frizzled* receptors and Wnt signaling in regulating

postnatal angiogenesis, although *Frizzled-2* is expressed in endothelial cells (38) and there is evidence suggesting Wnt signaling inhibits endothelial cell proliferation (39).

Gax Expression Blocks NF-κB Binding to its Consensus DNA-Binding Sequence

Given that NF-κB activity has been implicated in the changes in phenotype and gene expression endothelial cells undergo during angiogenesis caused by VEGF, TNF-α, and other factors (16–22), we wished to confirm our findings from gene expression profiling that *Gax* inhibits NF-κB activity in endothelial cells. We therefore did electrophoretic mobility shift assays with a probe containing an NF-κB consensus sequence (40) utilizing nuclear extracts from HUVECs transduced with either Ad.r*Gax* or the control adenoviral vector Ad.GFP. *Gax* expression in HUVECs markedly reduced specific binding to NF-κB consensus sequence by nuclear extracts compared with what was observed in controls (Fig. 6*A*), implying that *Gax* expression interferes with the binding of NF-κB to its consensus sequence. Unlabeled double-stranded NF-κB consensus oligonucleotide competed with labeled probe for binding (Fig. 6*B*), and random oligonucleotide and an NF-κB site with a point mutation that abolishes DNA binding (see Materials and Methods for sequences) failed to compete with the probe-specific band (data not shown).

Discussion

Interactions between tumors and their surrounding stroma, particularly the ability of tumors to induce angiogenesis, are critical to tumor progression and metastasis (41). At the endothelial cell level, the process of angiogenesis involves complex temporally coordinated changes in phenotype and global gene expression in response to alterations in the balance between pro- and antiangiogenic factors (2, 3). The stimuli for these changes are communicated from the surface of endothelial cells to the nucleus through multiple overlapping signaling pathways. The peptide factors and the receptors they bind to that activate these pathways have been the subject of intense study over the last decade, because the importance of aberrant endothelial cell activation and angiogenesis to the pathogenesis of not just cancer, but of other diverse human diseases, such as atherosclerosis, diabetic retinopathy, psoriasis, and others, has become more apparent (42). Because blocking aberrant angiogenesis has the potential to be an effective strategy to treat or prevent cancer and other angiogenesis-dependent diseases, understanding how downstream transcription factors integrate upstream signals from pro- and antiangiogenic factors to alter global gene expression and produce the activated, angiogenic phenotype, has become increasingly important.

Homeobox genes represent a class of transcription factors that, given their ubiquitous roles in controlling body plan formation during embryogenesis, organogenesis, cell proliferation and differentiation, and numerous other important cellular processes (5, 7), might be expected to be involved in either promoting or inhibiting the conversion of quiescent, unactivated endothelial cells to the activated, angiogenic phenotype. Indeed, several homeobox genes (*HOXA9EC*, *HOXB3*, *HOXB5*, *HOXD3*, *HOXD10*, and *Hex*) have already been implicated in this process (7, 43). We postulated that at least one additional homeobox gene, *Gax*, is also likely to play an important role in regulating endothelial cell angiogenesis. Consistent with its regulation in vascular smooth muscle cells, in endothelial cells, *Gax* is rapidly down-regulated by serum, proangiogenic, and proinflammatory factors (Figs. 1 and 2), and is able to inhibit endothelial cell migration *in vitro* (Fig. 3) and angiogenesis *in vivo* (Fig. 4). These observations led us to examine the mechanism by which *Gax* inhibits endothelial cell activation by examining global changes in gene expression due to *Gax*. In addition to observing that *Gax* up-regulates cyclin kinase inhibitors and down-regulates a number of proangiogenic factors, we also found that *Gax* inhibits the expression of NF-κB target

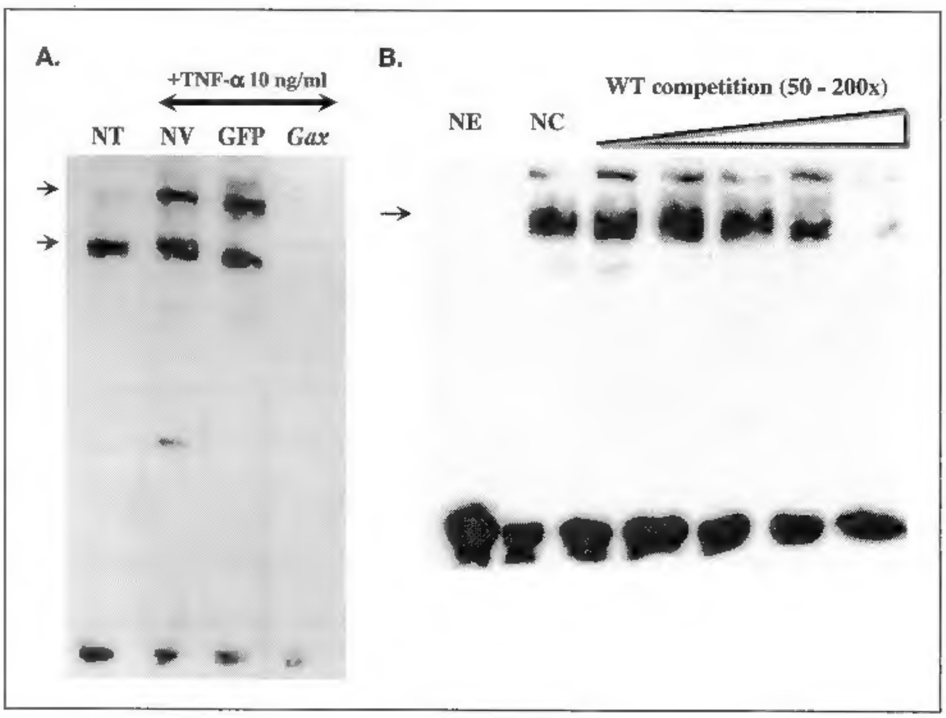

Figure 6. *Gax* expression inhibits NF-κB activity. *A*, *Gax* blocks NF-κB binding to its consensus sequence. HUVECs were infected with adenovirus containing GFP or r*Gax*, incubated overnight in EGM-2, and then induced with 10 ng/mL TNF-α for 1 hour. Controls were not induced with TNF-α. Nuclear extracts were prepared and incubated with biotinylated oligonucleotides containing the consensus NF-αB binding site (see Materials and Methods). *B*, control electrophoretic mobility shift assay. Excess unlabeled wild-type NF-κB oligonucleotide competes with NF-κB probe. Random oligonucleotide and an NF-κB site with a point mutation that abolishes DNA binding (see Materials and Methods for sequences) failed to compete with the probe-specific band (data not shown). Moreover, *Gax* expression did not affect binding to an unrelated probe (*Oct-1*, data not shown). *Arrows*, NF-κB specific bands, and *bands at the bottom of the gels* represent unbound probe. *NT*, no treatment with TNF-α; *NV*, no virus; *NE*, no nuclear extract; *NC*, no unlabeled competitor; and *WT*, wild-type.

genes (Table 1). Consistent with expression profiling data, *Gax* inhibits the binding of NF-κB to its consensus sequence (Fig. 6).

Several lines of evidence implicate NF-κB activity in regulating endothelial cell phenotype during inflammation and angiogenesis (16–19). For example, proangiogenic factors such as VEGF (33), TNF-α (44), and platelet-activating factor (17) can all activate NF-κB signaling and activity in endothelial cells. In addition, inhibition of NF-κB activity blocks tube formation *in vitro* on Matrigel (22), and pharmacologic inhibition of NF-κB activity suppresses retinal neovascularization *in vivo* in mice (45). Similarly, $\alpha_5\beta_1$-mediated adhesion to fibronectin also activates NF-κB signaling and is important for angiogenesis, and inhibition of NF-κB signaling inhibits bFGF-induced angiogenesis (16). One other potential mechanism by which NF-κB signaling may promote angiogenesis is through an autocrine effect, whereby activation of NF-κB induces expression of proangiogenic factors such as VEGF, as has been reported for platelet-activating factor–induced angiogenesis (17). Alternatively, the involvement of NF-κB in activating endothelial cell survival pathways is also likely to be important for sustaining angiogenesis (46).

Although NF-κB or IκB activity can regulate the expression of homeobox genes (47), there have been few reports of functional interactions between homeodomain-containing proteins and NF-κB or IκB proteins. The first such interaction reported was between IκBα and *HOXB7*, in which IκBα was reported to bind through its ankyrin repeats to the *HOXB7* protein and thus potentiate *HOXB7*-dependent gene expression (48). In contrast, the POU factor *Oct-1* can compete with NF-κB for binding to a specific binding site in the TNF-α promoter because its consensus sequence is close to the NF-κB consensus sequence (49). In addition, at least one interaction has been described in which a homeobox gene directly inhibits NF-κB-dependent gene expression, an interaction in which *Cdx2* blocks activation of the cyclooxygenase-2 promoter by binding p65/RelA (50). It remains to be elucidated if *Gax* inhibits NF-κB-dependent gene expression by a similar mechanism. Regardless of the mechanism, however, this report represents to our knowledge the first description of a homeobox gene that not only inhibits the phenotypic changes that occur in endothelial cells in response to proangiogenic factors but also inhibits NF-κB–dependent gene expression in vascular endothelial cells while doing so. These properties suggest *Gax* as a potential important transcriptional inhibitor of endothelial cell activation and thus a potential target for the antiangiogenic therapy of cancer or other angiogenesis-dependent diseases. In addition, understanding the actions of *Gax* on downstream target genes, signals that activate or repress *Gax* expression, and how *Gax* regulates NF-κB activity in endothelial cells is likely to lead to a better understanding of the mechanisms of tumor-induced angiogenesis and the identification of new molecular targets for the antiangiogenic therapy of cancer.

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